Occurrence and ecology of antibiotic Resistance determinants in wastewater Treatment systems

Pallares Vega, R.

DOI
10.4233/uuid:6a8cabb6-ce9b-42bf-a356-4be99e868cfe

Publication date
2021

Document Version
Final published version

Citation (APA)
Pallares Vega, R. (2021). *Occurrence and ecology of antibiotic Resistance determinants in wastewater Treatment systems*. [Dissertation (TU Delft), Delft University of Technology].
https://doi.org/10.4233/uuid:6a8cabb6-ce9b-42bf-a356-4be99e868cfe

Occurrence and ecology of antibiotic resistance determinants in wastewater treatment systems

REBECA PALLARÉS VEGA

Occurrence and ecology of antibiotic resistance determinants in wastewater treatment systems

Dissertation

For the purpose of obtaining the degree of doctor

at Delft University of Technology

by the authority of the Rector Magnificus, Prof.dr.ir. T.H.J.J. van der Hagen,

Chair of the Board for Doctorates

to be defended publicly on

Wednesday 10 November 2021 at 10:00 o´clock.

by

Rebeca PALLARES VEGA

Master of Science in Research and Advances in Microbiology

University of Granada, Spain

Born in Palencia, Spain.

This dissertation has been approved by the promotor

Composition of the doctoral committee:

Rector Magnificus,	chairperson
Prof.dr.ir. M.C.M. van Loosdrecht	Delft University of Technology, promotor
Dr.ir. D.G. Weissbrodt	Delft University of Technology, copromotor
Dr. H. Schmitt	Utrecht University, copromotor

Independent members:

Prof.dr. G.J. Medema	Delft University of Technology
Prof.dr. A.M. de Roda Husman	Utrecht University
Prof.dr.ir. D.W. Graham	Newcastle University, United Kingdom
Dr. C.M. Manaia	Universidade Católica Portuguesa, Portugal
Prof.dr.ir. M. de Kreuk	Delft University of Technology, reserve member

Dr.ir. L. Hernández Leal from Wetsus, European Centre of Excellence for Sustainable Water Technology, has supervised this work and contributed to the preparation of this dissertation.

This work was financially supported by The Source Separated Sanitation theme at Wetsus – European Centre of Excellence for Sustainable Water Technology in Leeuwarden, The Netherlands. Besides, this research has received funding from the European Union's Horizon2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 665874 and was co-funded by STOWA.

243 pages

Printed by	Proefschriftmaken
Cover design by	Lucía Antruejo (www.luciaantru.com)

ISBN 978-94-6423-473-2
An electronic version of this dissertation is available at http://repository.tudelft.nl/.

Para mi familia.

Para mi abuelo Paco,
que me vio empezar pero ya no está aquí para verme terminar.
Sé que te hubiese encantado tocar estas páginas.

Table of Contents

Summary

The rise of antibiotic resistant bacteria threatens the existing *status quo* of successful treatment of infectious diseases, leading to substantial personal and economic losses.

Wastewater, carrying antibiotic resistant microorganisms from fecal origin, is an important route for disseminating anthropogenic-related resistant bacteria to natural ecosystems. Wastewater treatment plants (WWTPs), collecting and treating sewage, comprise an opportunity to mitigate such dissemination. However, because of their intrinsic characteristics, namely constant nutrient inputs, presence of selectors in sewage (*i.e.,* antibiotics), and high bacterial densities within the biological treatment, these facilities have been postulated as environments selecting for antibiotic resistant bacteria and fostering horizontal exchange of antibiotic resistance genes (ARGs).

Unravelling the ecology of antibiotic resistant determinants in WWTPs is essential to identify which stages or technologies are critical for their proliferation or removal and pinpoint possible additional or alternative intervention strategies. This thesis aims to contribute to such a quest with a multidimensional approach. The work presented here involves extensive field studies combined with qPCR measurements and statistical analysis to assess how WWTPs affect antibiotic resistant determinants. In addition, culture and molecular assays are used to investigate the conjugal exchange of plasmid-borne antibiotic resistance in wastewater environments.

In the two field investigation chapters (Chapters 2 and 3), the prevalence patterns of antibiotic residues and resistance determinants (ARGs and mobile genetic elements) are measured throughout (Dutch) WTTPs. In Chapter 2, resistance determinants are evaluated across more than 60 facilities in one single point (cross-sectional). In Chapter 3, antibiotic residues and gene determinants are monitored monthly for a year across three selected WWTPs with different treatments. Most importantly, these two chapters provide a comprehensive evaluation of the influence of diverse factors (ranging from the design and operation of WWTPs to abiotic components) on the dynamics of resistance determinants. The results obtained offer quantitative data on the occurrence, removal, and discharge levels of both antibiotic residues and gene determinants. Altogether, these findings support that conventional WWTPs (mainly via biological treatment) reduce the concentration of antibiotics (10-100%) and ARGs in wastewater (on average ca.1.5-2.5 logs), lowering their dissemination towards the environment.

Still, gene determinants and some antibiotics are currently discharged with the effluent above the levels present in the receiving freshwaters. The data gathered here may contribute to calculating the possible associated risk for human exposure and antibiotic resistance proliferation in impacted freshwaters ecosystems (Chapter 6). Our results also showed that no specific treatment design or technology (i.e., presence/absence of primary clarifier or treatment based on flocculent or granular activated sludge) comprised and advantage in the removal of gene determinants. Notably, increased volumetric loading had a significant detrimental effect on the ARGs removal capacity (removal was reduced by an average 0.4 logs per time the average flow was doubled). These findings are of great interest from a surveillance perspective and highlight a possible improvement point within the treatment. An additional critical point was related to effluent suspended solids since poorer removals of gene determinants were correlated with higher effluent turbidity. An improved reduction of ARG during rain events might therefore be achieved by better management of solid/liquid separation processes.

In the wet-lab experimental chapters (Chapters 4 and 5), the horizontal gene transfer of ARGs is addressed. Plasmid borne dissemination of ARGs in wastewater environments is of concern as indigenous sludge microbiota could act as sink and reservoir of anthropogenic antibiotic resistance. The dynamics of conjugal transfer of plasmids have been studied for decades, but mostly under optimal laboratory conditions. Thus, many aspects of the ecology of plasmids exchange in complex natural and engineered environments remain obscure. Chapters 4 and 5 provide quantitative information on the influence of relevant parameters (temperature, nutrient levels and redox conditions) on the frequency of transfer events in wastewater environments. An IncP-1 plasmid, a plasmid family highly prevalent in both wastewater and biosolids (Chapter 2 and 3), was used as a vector for both the *in vitro* (Chapter 4) and *in situ* (Chapter 5) conjugation assays. Our findings *in vitro* suggested that typical psychrophilic temperatures in wastewater environments (9 – 15º C) still support the conjugal transfer of plasmids. Contrarily, conjugation appeared to be hindered by low nutrient conditions, such as those present in soil or wastewater. The latter deserves further attention as it may constitute a new strategy for preventing or lowering conjugation. Finally, *in situ* assays proved that culture-independent quantification of transconjugants from sludge microcosms is possible. The combination of fluorescently labelled strains and flow cytometry detection of transconjugant cells was successful in quantifying transconjugants under optimal conditions (aerobic and 30ºC). However, detection limits of the given method might be a problem when evaluating sub-optimal mating conditions (i.e. psychrophilic temperatures).

Collectively, this thesis shows that conventional WWTPs reduce the antibiotics and ARGs emissions towards surface waters, although their removal capacity is hindered by increasing water volumes and effluent suspended solids. A more realistic evaluation of the potential conjugal exchange showed that it is possible under the condition present in wastewater environments (temperature and redox) although it might be limited by the low nutrient availability.

Samenvatting

De opkomst van antibiotica-resistente bacteriën vormt een bedreiging voor de bestaande *status quo* van succesvolle behandeling van infectieziekten, wat kan leiden tot aanzienlijke persoonlijke en economische verliezen.

Afvalwater, dat antibiotica-resistente micro-organismen van fecale oorsprong bevat, is een belangrijke route voor de verspreiding van antropogeen gerelateerde resistente bacteriën naar natuurlijke ecosystemen. Afvalwaterzuiveringsinstallaties (AWZI's), die afvalwater inzamelen en behandelen, bieden een mogelijkheid om een dergelijke verspreiding tegen te gaan. Wegens de intrinsieke proceseigenschappen, namelijk een constante toevoer van nutriënten, de aanwezigheid van een selectiedruk in het rioolwater (d.w.z. antibiotica) en hoge bacteriële dichtheden, zou een biologische zuiveringsinrichting een omgevingen kunnen zijn die selecteert op antibioticaresistente bacteriën en horizontale uitwisseling van antibioticaresistentiegenen (ARG's) bevorderd.

Het ontrafelen van de ecologie van antibiotica-resistente determinanten in AWZI's is essentieel om te identificeren welke stadia of technologieën kritisch zijn voor hun proliferatie of verwijdering en om mogelijke bijkomende of alternatieve interventiestrategieën te bepalen. Deze dissertatie wil hiertoe bijdragen met een multidimensionele benadering. Het hier gepresenteerde werk omvat een uitgebreide veldstudies in combinatie met qPCR-metingen en statistische analyse om na te gaan hoe antibioticaresistente determinanten zich in AWZI's gedragen. Daarnaast zijn kweek- en moleculaire assays gebruikt om de conjugale uitwisseling van plasmide-gebaseerde antibioticaresistentie in afvalwatersystemen te onderzoeken.

Het veldonderzoek (Hoofdstuk 2 en 3) worden de prevalentiepatronen van antibioticaresiduen en resistentiedeterminanten (ARG's en mobiele genetische elementen) gemeten in (Nederlandse) RWZI's beschreven. In hoofdstuk 2 worden resistentie determinanten geëvalueerd voor meer dan 60 AWZI's middels één bemonstering (cross-sectioneel) beschreven. In hoofdstuk 3 worden antibiotica-residuen en resistentie determinanten gedurende een jaar maandelijks gemonitord in drie geselecteerde RWZI's met verschillende procesontwerpen beschreven. Het belangrijkste is dat deze twee hoofdstukken een uitgebreide evaluatie geven van de invloed van diverse factoren (variërend van het ontwerp en de werking van RWZI's tot abiotische componenten) op de dynamiek van resistentiebepalende stoffen. De verkregen resultaten bieden kwantitatieve gegevens over het vóórkomen, de verwijdering en

de lozingsniveaus van zowel antibioticaresiduen als resistentie-genen Al met al ondersteunen deze bevindingen dat conventionele RWZI's (voornamelijk via biologische zuivering) de concentratie van antibiotica (10-100%) en ARG's in afvalwater verminderen (gemiddeld ca. 1,5-2,5 logs), waardoor de verspreiding ervan naar het milieu wordt verminderd. Toch worden nog steeds ARG's en sommige antibiotica met het effluent geloosd boven de niveaus die aanwezig zijn in het ontvangende oppervlaktewater. De hier verzamelde gegevens kunnen bijdragen tot de berekening van het mogelijke risico van blootstelling van de mens en de verspreiding van antibioticaresistentie in de beïnvloede zoetwaterecosystemen (hoofdstuk 6). Onze resultaten toonden ook aan dat een specifiek behandelingsontwerp of -technologie (d.w.z. aan- of afwezigheid van een voorbezinking of behandeling middels vlokkig of korrelig actief slib) niet direct resulteerde in een verschillende verwijdering van resistentie genen stoffen. Vooral een hoge volumetrische belasting (korte verblijftijd) had een aanzienlijk nadelig effect op het verwijderingsvermogen van de ARG's (de verwijdering daalde met gemiddeld 0,4 log per keer dat het gemiddelde debiet verdubbeld). Deze bevindingen zijn van groot belang vanuit een bewakingsperspectief en wijzen op een mogelijk verbeterpunt binnen de behandeling. Een bijkomend kritiek punt had betrekking op de gesuspendeerde vaste stoffen in het effluent, aangezien een slechtere verwijdering van resistentie genen gecorreleerd was met een hogere troebelheid van het effluent. Een betere verwijdering van de resistentie genen tijdens regenval zou daarom kunnen worden bereikt door een beter beheer van de vast-vloeistof scheidingsprocessen.

In de hoofdstukken over laboratorium experimenten (hoofdstukken 4 en 5) wordt de horizontale genoverdracht van ARGs behandeld. Plasmide gedragen verspreiding van ARGs in afvalwater omgevingen is van belang omdat natuurlijk microorganismen kunnen fungeren als reservoir van antropogene antibioticaresistentie. De dynamiek van de conjugale overdracht van plasmiden wordt al tientallen jaren bestudeerd, maar meestal onder optimale laboratoriumomstandigheden. Bijgevolg blijven vele aspecten van de ecologie van plasmidenuitwisseling in complexe natuurlijke en reactor omgevingen onduidelijk. De hoofdstukken 4 en 5 geven kwantitatieve informatie over de invloed van relevante parameters (temperatuur, nutriëntenniveaus en redoxcondities) op de frequentie van transfergebeurtenissen in afvalwatermilieus. Een IncP-1 plasmide, een plasmidefamilie die veel voorkomt in zowel afvalwater als actiefslib (Hoofdstuk 2 en 3), werd gebruikt als vector voor zowel de in-vitro (Hoofdstuk 4) als in-situ (Hoofdstuk 5) conjugatie assays. Onze in-vitro bevindingen suggereerden dat typische psychrofiele temperaturen in afvalwater omgevingen (9

- 15º C) nog steeds de conjugale overdracht van plasmiden ondersteunen. De conjugatie bleek daarentegen te worden belemmerd door omstandigheden met een laag nutriëntengehalte, zoals die in grond of gereinigd afvalwater voorkomen. Dit laatste verdient verdere aandacht aangezien het een nieuwe strategie kan vormen om conjugatie te voorkomen of te verminderen. Tenslotte toonden in-situ assays aan dat cultuuronafhankelijke kwantificering van transconjuganten uit slib-microkosmos experimenten mogelijk is. De combinatie van fluorescent gelabelde stammen en flowcytometrische detectie van transconjugerende cellen was succesvol in het kwantificeren van transconjuganten onder optimale omstandigheden (aëroob en 30ºC). De detectiegrenzen van de gegeven methode kunnen echter een probleem vormen bij de evaluatie van suboptimale paringsomstandigheden (d.w.z. psychrofiele temperaturen).

Samenvattend toont dit proefschrift aan dat conventionele RWZI's de emissie van antibiotica en ARG's naar het oppervlaktewater verminderen, hoewel hun verwijderingscapaciteit wordt belemmerd door toenemende watervolumes en gesuspendeerde vaste stoffen in het effluent. Een meer realistische evaluatie van de potentiële conjugale uitwisseling toonde aan dat deze mogelijk is onder de omstandigheden aanwezig in afvalwateromgevingen (temperatuur en redox), hoewel deze beperkt kan worden door de lage beschikbaarheid van nutriënten. Een beter inzicht van dit proces zal helpen om .

Resumen

El aumento de las bacterias resistentes a los antibióticos entraña una amenaza en el actual *statu quo* en el tratamiento de enfermedades infecciosas, causando importantes pérdidas personales y económicas.

Las aguas residuales constituyen una importante vía de diseminación de bacterias resistentes de origen antropogénico a los ecosistemas naturales, ya que que albergan microorganismos resistentes a los antibióticos de origen fecal. Las plantas de tratamiento de aguas residuales (EDAR), que recogen y tratan las aguas residuales, suponen una oportunidad para mitigar dicha diseminación. Sin embargo, debido a sus características intrínsecas, es decir, al constante aporte de nutrientes, la presencia de agentes de selección (p.e. antibióticos) y las altas densidades bacterianas dentro del tratamiento biológico, se ha postulado que estas instalaciones son entornos que seleccionan a las bacterias resistentes a los antibióticos y fomentan el intercambio horizontal de genes de resistencia a los antibióticos (ARGs por sus siglas en inglés ¨antibiotic reistance genes¨).

Desentrañar la ecología de los determinantes de la resistencia a los antibióticos en las EDAR es clave para identificar qué etapas del tratamiento o qué tecnologías son críticas para su proliferación o eliminación, así como señalar posibles estrategias de intervención adicionales o alternativas. Esta tesis pretende contribuir a dicha búsqueda con un enfoque multidimensional. El trabajo aquí dispuesto incluye amplios estudios de campo combinados con métodos moleculares (reacción de la polimerasa cuantitativa (qPCR)) y análisis estadísticos para evaluar cómo afectan las EDAR a los determinantes de resistencia a los antibióticos. Además, se utilizan ensayos de cultivo y moleculares para investigar el intercambio de la resistencia a los antibióticos mediada por plásmidos (conjugación) en aguas residuales.

En los dos capítulos basados en muestreo de campo (capítulos 2 y 3), se evalúan los patrones de prevalencia de los residuos de antibióticos y los determinantes de resistencia (ARGs y elementos genéticos móviles) en multitud de EDAR (en los Países Bajos). El capítulo 2 comprende un estudio transversal en el que se cuantifican los determinantes de resistencia en más de 60 instalaciones pero sólo una vez por instalación. En el capítulo 3, los residuos de antibióticos y los determinantes genéticos se miden mensualmente durante un año en tres EDAR específicas que cuentan con diferentes tratamientos. Conjuntamente, estos dos capítulos proporcionan una evaluación exhaustiva de la influencia de diversos factores (que van

desde el diseño y el funcionamiento de las EDAR hasta los componentes abióticos) en la dinámica de los determinantes de resistencia. Los resultados obtenidos ofrecen datos cuantitativos sobre la aparición, eliminación y los niveles de emisión tanto de los residuos de antibióticos como de los determinantes genéticos. En conjunto, estos resultados apoyan que las EDAR convencionales (principalmente a través del tratamiento biológico) reducen la concentración de antibióticos (10-100%) y ARGs en las aguas residuales (en promedio ca.1.5-2.5 logs), disminuyendo su diseminación hacia el medio ambiente. Aun así, los determinantes genéticos y algunos antibióticos son constantemente vertidos junto con el efluente en niveles superiores a los presentes en las aguas receptoras. Los datos aquí recogidos pretenden contribuir tanto al cálculo del posible riesgo de proliferación los determinantes de resistencia en los ecosistemas afectados, como a la estimación del riesgo para la salud de la población al exponerse a dichas aguas que contienen los citados agentes (capítulo 6). Nuestros resultados también demuestran que ningún diseño o tratamiento específico (p.e. presencia/ausencia de clarificador primario o tratamiento basado en lodos activados floculantes o granulares) supone una mejora en la eliminación de genes de resistencia. Sin embargo, sí observamos que el aumento de la carga volumétrica conlleva un efecto perjudicial significativo en la capacidad de eliminación de ARGs (la eliminación se reduce en una media de 0,4 logs por cada vez que se duplica el caudal medio). Estos resultados son de gran interés para las campañas de monitoreo de aguas residuales y ponen de manifiesto un posible punto de mejora dentro del tratamiento. Otro punto crítico parece ser la cantidad de sólidos en suspension en el efluente, ya que encontramos que la eliminación de los determinantes genéticos es inversamente proporcional a la turbidez del efluente. Sugerimos, por tanto, que una mejor gestión de los procesos de separación de las fases sólido/líquido podría mejorar la reducción de ARGs durante los periodos de lluvia.

En los capítulos experimentales de laboratorio (capítulos 4 y 5), se aborda la transferencia horizontal de los ARG. La diseminación de plásmidos que contienen ARGs en aguas residuales y ecosistemas afines preocupa, ya que la microbiota autóctona de los lodos podría actuar como sumidero y reservorio de la resistencia a los antibióticos de origen antropogénico. La dinámica de la transferencia de plásmidos por conjugación se ha estudiado durante décadas, pero estos estudios se han basado en su mayoría en condiciones óptimas de laboratorio. De este modo, muchos aspectos de la ecología del intercambio de plásmidos en entornos naturales (y artificiales) complejos siguen siendo desconocidos. Los capítulos 4 y 5 proporcionan información cuantitativa sobre la influencia de parámetros relevantes

(temperatura, niveles de nutrientes y condiciones redox) en la frecuencia de los eventos de conjugación en ecosistemas de aguas residuales. Para estos experimentos, empleamos un plásmido IncP-1, una familia muy frecuente tanto en las aguas residuales como en los biosólidos (capítulos 2 y 3). Este tipo de plásmidos es también muy usado como vector en los ensayos de conjugación *in vitro* (capítulo 4) e *in situ* (capítulo 5). Nuestros resultados *in vitro* sugieren que las temperaturas psicrófilas, típicas de los entornos de aguas residuales (9 - 15º C), siguen favoreciendo los eventos de conjugación. Por el contrario, la conjugación parece verse obstaculizada en situaciones con bajos niveles de nutrientes, como las presentes en el suelo o en las aguas residuales. Estos resultados merecen especial atención, ya que puede constituir una nueva estrategia para prevenir o disminuir los eventos de conjugación. Por último, los ensayos *in situ* en microcosmos de lodos activos demostraron que es posible la cuantificación de transconjugantes con técnicas independientes del cultivo. La combinación de cepas con marcadores fluorescentes y la detección por citometría de flujo logró la cuantificación de transconjugantes en condiciones óptimas (aeróbicas y 30ºC). Sin embargo, los límites de detección del método dado podrían ser un problema cuando se evalúan condiciones subóptimas para las bacterias empleadas (es decir, temperaturas psicrófilas).

CHAPTER 1

1

General Introduction

1.1 Antibiotics and antibiotic resistance.

Antibiotics are chemotherapeutic substances capable of eliminating bacteria or inhibiting their growth. This is different from antimicrobials, a broader term that includes substances inhibiting the growth of bacteria or other microorganisms (fungi, viruses, and/or parasites). Antibiotics disrupt the cellular processes of the target cell by different modes of action. Some of the most common are: ❶ disruption of cell wall synthesis, ❷ inhibition of the folic acid metabolic pathway, ❸ inhibition of DNA or RNA synthesis, or ❹ inhibition of protein synthesis by binding to either 30S or 50S ribosomal subunits (Kapoor et al., 2017, **Figure 1.1**).

The use of antibiotics is considered a milestone in the treatment of infectious diseases. This medical revolution started with the discovery and widespread use of penicillin in the first half of the 20th century. Many other natural substances were identified as antibiotics after penicillin during the so-called 'golden era of antibiotics' (1950-70). In the following decades, several antibiotic drugs have been obtained mainly by chemical synthesis and modification of the pre-existing substances (Aminov, 2010). Unfortunately, despite the increasing efforts, a limited number of new compounds have been commercialized in the past 20 years (Aminov, 2010). Information about some of the most commonly prescribed antibiotics (Nethmap-Maran, 2018), along with their mechanisms of action, is summarized in **Table 1.1**. This selection includes the antibiotic for which residues in wastewater were analysed in **Chapter 3** of this thesis.

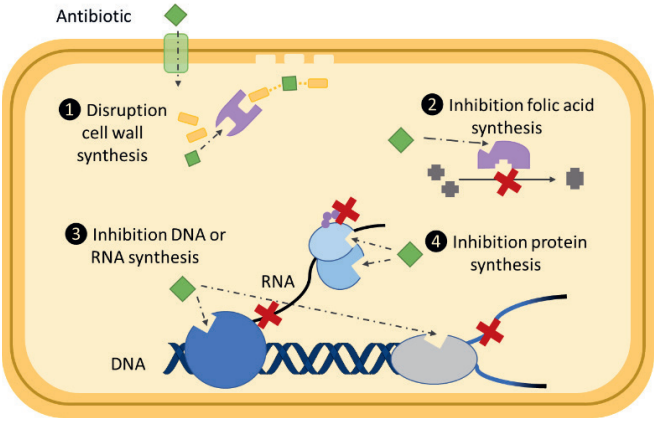


Figure 1.1. Main antibiotic targets within the bacterial cell. Adapted from Allen et al., 2010

Alongside the scarcity of new antibiotics, a more significant threat to the use of these therapeutic agents has arisen in the last years: the increasing numbers of target bacteria resistant to them (WHO, 2014).

Antibiotic resistance is defined as the natural ability of certain bacteria to bypass the bacteriostatic or bactericidal effect of a particular antibiotic. Encoded by the so-called antibiotic resistance genes (ARGs), bacteria have developed numerous mechanisms to evade the action of antibiotics. These mechanisms can be classified into four main groups (**Figure 1.2**): ❶ inhibition of the antibiotic uptake, ❷ expulsion of the antibiotic by efflux pumps ❸ antibiotic inactivation by enzymes, ❹ modification of the antibiotic target (Allen et al., 2010). A myriad of different mechanisms can result in resistance to the same class of antibiotic. As a result, ARGs are generally classified according to the antibiotic towards which they confer resistance rather than by their mechanism of action. Some examples of ARGs and their mechanisms of action are displayed in **Table 1.1**. These are only a small fraction of the total number of known ARGs, which is continuously increasing. Since it is impossible to examine all ARGs, defining which surrogate ARGs are best to monitor antibiotic resistance is essential. This decision should account for the scope of the study (relevant to the environment/clinical setups) and the expected prevalence (new clinically relevant ARGs often lay below detection limits - ca. 100 copies mL^{-1} - in the natural environment). An example of a selection panel of surrogate ARGs was recently proposed by Berendonk et al. (2015). The ARGs studied in **Chapters 2 and 3** of this thesis are based on Berendonk´s selection.

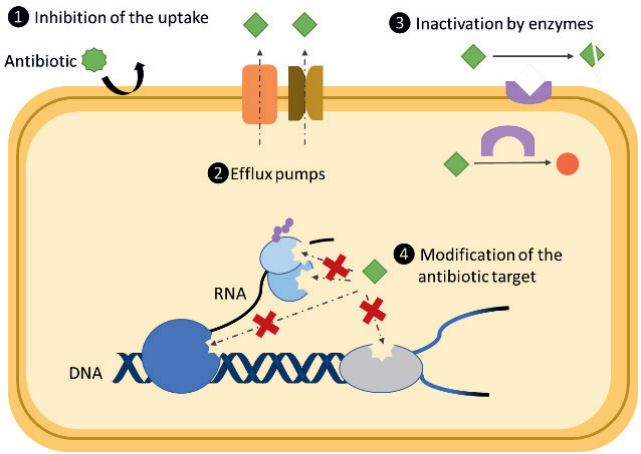


Figure 1.2. Main antibiotic resistance mechanisms in a Gram-negative bacterial cell. Adapted from Allen et al., 2010.

Table 1.1. Main currently available antibiotics classes and mode of action, ARGs, and resistance mechanisms. Abbrev: MSL: Macrolides Streptomycin and Lincosamides.

Antibiotic Class	Example	Mode of action (Inhibition/disruption of)	Antibiotic Resistance class	ARG	Resistance mechanism
Aminoglycosides	Gentamycin	Protein synthesis (binding to 50S ribosomal subunit)	Aminoglycoside resistance	*aadA1*	Drug inactivation
				strB	Drug inactivation
β – lactams	Amoxicillin	Cell wall synthesis	β – lactamases	*ampC*	Drug inactivation
Penicillins	Ampicillin				
	Penicillin				
Cephalosporines	Cefotaxime	Cell wall synthesis	Extended spectrum	bla_{SHV32}	Drug inactivation
			β – lactamases (ESBL)	bla_{CTXM}	Drug inactivation
Carbapenems	Meropenem	Cell wall synthesis		bla_{KPC}	Drug inactivation
				bla_{OXA-48}	Drug inactivation
Glycopeptides	Vancomycin	Cell wall synthesis	Vancomycin resistance	*vanA*	Mod. antibiotic target
Macrolides	Azithromycin	Protein synthesis	Erythromycin resistance	*ermB, ermF*	Mod. antibiotic target
	Clarithromycin	(binding to 50S ribosomal	Macrolides resistance	*mphA, mphB*	Drug inactivation
	Erythromycin	subunit)		*mefA*	Antibiotic target protection
Lincosamides	Clindamycin	Protein synthesis	MSL resistance	*ermA*	Mod. antibiotic target
Phenicols	Chloramphenicol	Protein synthesis (binding to 50S ribosomal subunit)	Chloramphenicol resistance	*cat*	Drug inactivation
Quinolones	Ciprofloxacin	DNA synthesis	Quinolones resistance	*qnrS*	Antibiotic target protection
	Norfloxacin	(binding to DNA gyrase)		*aac(6')-ib-cr*	Drug inactivation
Sulphonamides &	Sulfamethoxazole	Folic acid synthesis	Sulphonamides resistance	*sul1, sul2*	Mod. antibiotic target
trimethoprim	Sulphapyridine		Trimethoprim resistance	*dfrA1*	Mod. antibiotic target
	Trimethoprim				
Tetracyclines	Tetracycline	Protein synthesis	Tetracycline resistance	*tetM, tetW*	Antibiotic target protection
	Doxycycline	(binding to 30S ribosomal subunit)		*tet30*	Efflux pump

1.2. Rise of antibiotic resistance as a growing global issue.

Although attention for antibiotic resistance has increased in the last decade, it is not a recent phenomenon, and it is not exclusively associated with human activities. ARGs have been detected in microorganisms isolated from pristine environments dating back thousands of years (D'Costa et al., 2011). Specific antibiotic resistant mechanisms are thought to have originated millions of years ago (Fevre et al., 2005). This should not be surprising since antibiotic substances are commonly produced by natural environmental microorganisms such as fungi (Cook and Lacey, 1945; Xu et al., 2015) or bacteria (Baltz, 2008; Weber et al., 2003).

In natural ecosystems, the production of antibiotics may be related to cell-signalling and communication rather than inhibition of the growth of possible competitors (Aminov, 2009). In any case, both the antibiotic producers (Cundliffe, 1989) and the other microorganisms co-existing in those environments have often developed resistant mechanisms through mutations in their genome to evade or cope with the effect of the antibiotics. Mostly, these are spontaneous events (mutation rates in bacteria occur at 0.003 per genome per generation (Drake et al., 1998)). However, they can also be induced *de novo* or increased under stress conditions triggers such as antibiotics presence (Galhardo et al., 2007). Because of the competitive advantage they confer, mutations that encode antibiotic resistance have been subsequently maintained and spread to the offspring of those microorganisms by vertical transfer, confirming the intrinsic resistome of different bacteria species. In general, these resistant bacteria are mainly harmless environmental species, and only a few taxa (i.e. *Pseudomonas, Acinetobacter*) might represent a real threat to human health (Davies and Davies, 2010). Yet, antibiotic resistance as a global hazard is not primarily caused by the vertical transfer of intrinsic resistance determinants in environmental species. It arises from the exchange of the natural species-specific ARGs towards other taxa, including potential pathogens for human health, by a mechanism known as horizontal gene transfer (HGT; explained in detail in the next section).

With the intensification of antibiotic use in the 20th century, novel ARGs emerged and were horizontally exchanged, leading to a vast and diverse amount of resistant bacteria, including multi-resistant strains causing untreatable infections (Wright, 2007). Consequently, antibiotic resistance has become a concerning cause of mortality, accounting for more than 33.000 annual deaths only in the European Economic Area (Cassini et al., 2019). The economic consequences of this problem are also of great concern. In 2007, the European Centre for Disease and Control estimated a 1.5 billion € per year as costs derived from antibiotic resistant

infections. These included clinical expenses (extended hospital stays) and also indirect costs (i.e. productivity loss due to medical leaves) in the European Economic Area (ECDC/EMEA Joint Working Group., 2009). In other parts of the globe where antibiotics are still used as growth promoters and/or sold without prescription over the counter, antibiotic resistance impact might be even more significant (Laxminarayan et al., 2015; Sartelli et al., 2020). Since bacteria do not respect country borders, a global perspective to tackle antibiotic resistance is fundamental (Hernando-Amado et al., 2020).

1.3. Horizontal gene transfer

Unlike vertical gene transfer, which comprises genetic material inheritance from parent cells to offspring, HGT may involve exchanging genes across different bacterial populations and, often, across distant phylogenetic taxa. This mechanism compensates for the lack of sexual reproduction in Bacteria (as occurs in eukaryotic cells), promoting genetic diversity and adaptation. Therefore, HGT drives evolution in bacterial populations (Ochman et al., 2000).

The main mechanisms of HGT are transformation, transduction, and conjugation, depicted in **Figure 1.3.**

Transformation is the ability of bacteria to take up free extracellular double-stranded DNA (that originates from active secretion or cell lysis) and incorporate it into their genome. This mechanism does not require close contact with other cells but involves a specific physiological state of the cells known as competency. Transformation has been demonstrated in various natural environments such as marine and ground waters, rivers and soil (Davison, 1999). Yet, only a restricted fraction of species (~80) widespread across diverse species are known to be naturally competent (Johnston et al., 2014). The majority of these naturally competent bacteria undergo the competent state for short periods of time in response to stress-related circumstances such as cell density or starvation. In addition, extracellular DNA might be subjected to quick degradation by nucleases, albeit its association to some mineral particles (i.e. clay) might prolong its persistence in the environment (Nielsen et al., 2007). These characteristics might have contributed to overlooking the relevance of transformation in HGT events, including related to the spread of ARGs (Woegerbauer et al., 2020). However, a new interest in re-evaluating the role of extracellular DNA and the importance of transformation is arising recently (Hasegawa et al., 2018; Mao et al., 2014).

Transduction is the exchange of genetic information mediated by viral vectors: bacteriophages. Transduction occurs when bacterial genetic information is inadvertently packed together with the virus genome into the phage capsid. Bacteriophages are abundant in aquatic environments (Bergh et al., 1989) where high transduction frequencies have been observed (Kenzaka et al., 2010). Genetic traits from phage origin are commonly found in bacteria, and bacteria genes (including ARGs) are ubiquitous in the phage fraction of environmental samples (Calero-Cáceres et al., 2014; Colomer-Lluch et al., 2011). However, the contribution of transduction to HGT of antimicrobial resistance genes is thought to be restricted by the narrow host range of bacteriophages, limiting the genetic exchange to phylogenetically close related bacteria, mainly at the species or genus level (Popa et al., 2017).

Conjugation is the exchange of genetic material by a cell to cell contact through a cell pore. Conjugation was first detected in *Escherichia coli* by means of its fertility plasmid "F factor" (Lederberg, 1952; Lederberg et al., 1952) and nowadays is known to be mainly driven through plasmid and integrative conjugative elements (Smillie et al., 2010). Conjugation might occur at high frequencies (10^{-1} transconjugant per donor Thomas and Smith, 1987)) and has been demonstrated among diverse taxa including different kingdoms (Bates and Wilkins, 1998) and a wide variety of natural environments such as soil (Richaume et al., 1992), river (Muela et al., 1994) and seawater (Dahlberg et al., 1998). As a result, conjugation is considered as the principal mechanism of exchanging genetic elements, including those containing ARGs (Halary et al., 2010).

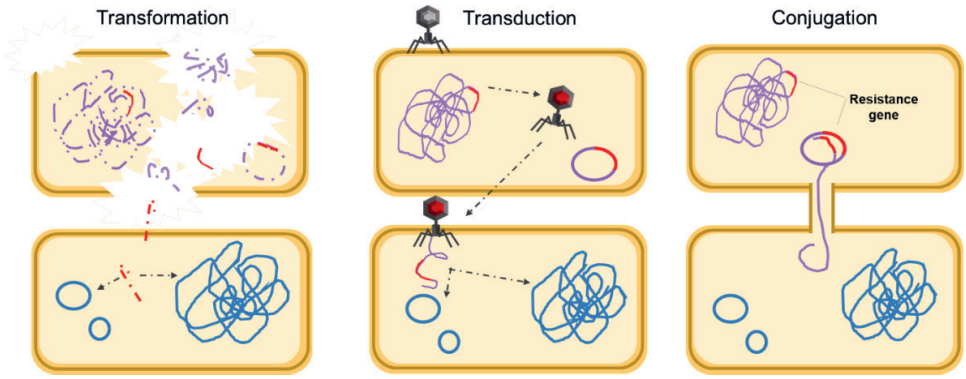


Figure 1.3. Horizontal gene transfer mechanisms in gram-negative bacteria.

1.4. Plasmids: drivers for the dissemination of antibiotic resistance.

Plasmids are circular double-stranded DNA molecules that can replicate independently from the chromosome. Plasmids vary in size (1-400 kb) and copy number (1-1000) and usually encode for accessory elements to the bacterial chromosome that confer a selective advantage to the host cell. Plasmid genes often encode secondary catabolic routes, virulence factors, and resistance to possible hazardous substances to the host cell, such as antibiotics, heavy metals, and or quaternary ammonium compounds. Plasmids regularly contain other MGEs, namely insertion sequences, transposons, and integrons, which allow them to incorporate genes from the chromosome or other plasmids co-existing in the host cell. In this manner, antibiotic resistance genes initially present in the chromosome of particular species or linages can be mobilized to the plasmid and then be shared across diverse other taxa by conjugation.

Not all plasmids contribute to the same extent in the exchange and dissemination of genetic traits. The transferability of plasmids depends on the presence of an oriT and other *mob* genes (relaxases and type IV couple gene) along with the mating pair formation *(mpf)* genes which encode for a type IV secretion system (*T4SS*), **Figure 1.4**. Conjugative plasmids (that can self-transfer) hold all the genes mentioned above. In contrast, mobilizable plasmids (that can only transfer by the presence of a conjugative plasmid, "helper") lack the *mpf* gene and need another plasmid (helper) within the cell to produce it for them. Mobilizable plasmids are often deficient in some of the mob genes as well (Smillie et al., 2010). Both conjugative and mobilizable plasmids are the leading agents in HGT events mediated by conjugation. The third group of plasmids, known as non-conjugative, lack all the main components mentioned above and can neither self-transfer nor be mobilized by a helper plasmid. Thus, their contribution to horizontal genetic exchange is expected to be negligible.

Figure 1.4. is removed from this version of the thesis for copyright reasons

Figure 1.4. Conjugative and mobilizable plasmids classification according to the presence/absence of mobilization (mob) and mating pair formation (mpf) genes. Taken from Smillie et al., 2010.

Plasmids can also be classified by their host range (narrow or broad), which is a qualitative term to describe their ability to be transferred and maintained in bacteria that are phylogenetically distant (Suzuki et al., 2010). Plasmids are classified into incompatibility (Inc) groups as well. This classification is based on their inability to persist and replicate within the same cell than other plasmids (Couturier et al., 1988; Datta and Hedges, 1971) when they harbour similar replication and partitioning systems. Incompatibility was first experimentally demonstrated by classical co-culture methods, yielding 23 phenotypically different types and many other subgroups. New molecular approaches, as the replicon typing method based on PCR targets (Carattoli et al., 2014), have allowed them to include novel groups and re-organize the former ones based on genetic similarities (Rozwandowicz et al., 2018).

Incompatibility group and host rage are sometimes associated: plasmids belonging to certain Inc groups (IncF, IncI, and IncK) are linked with narrow host range (i.e. transmission within Enterobacteriaceae family) while those belonging to the incompatibility groups IncN, IncP-1, IncQ, and IncW are considered broad host range (*i.e.* can be transferred from Enterobacteriaceae to Pseudomonadales (Dröge et al., 2000)). ARGs have been detected in most plasmid groups (Rozwandowicz et al., 2018) irrespective of their incompatibility type or host range. However, the contribution of the different plasmid groups to the dissemination resistant determinants is diverse depending on the context. Narrow host range plasmids (IncF, IncI, and IncK), highly prevalent in ubiquitous microorganisms in the gut such as Enterobacteriaceae, are responsible for the propagation of ARGs across clinical and animal production environments (Rozwandowicz et al., 2018). On the other hand, broad host range plasmids, as IncP-1 plasmids have been found in clinical isolates, but also in bacteria from soil and water. Thus, they are considered the main vectors in widespread resistant determinants in natural environments (Popowska and Krawczyk-Balska, 2013). Specific plasmids belonging to IncP-1 and IncI groups will be used in **Chapter 4** and **Chapter 5** of this thesis.

1.5. Environmental dissemination of anthropogenic antibiotic resistance.

Since their discovery and for many decades, antibiotic resistance has enormously increased in human healthcare-related settings (including clinical and community), and animal production environments where antibiotics were (and in occasions still are) intensively applied (Shallcross and Davies, 2014; Teuber, 2001). Of course, this is dependent on antibiotics prescription rates, which varies largely across countries (ESAC-Net, 2017). As previously mentioned, high antibiotic selective pressure contributes to selecting resistant bacteria, the

dissemination of their resistance determinants, and the rapid emergence of new resistant genes (Galhardo et al., 2007). Along with the alarming clinical consequences of such events, the environmental dissemination of resistance genes from the anthropogenic origin is a matter of increasing concern (Singer et al., 2016).

Consumed antibiotics are not fully metabolized and end up excreted (30-90%) with the faeces and urine in both animals and humans (Du and Liu, 2012). Along with these chemical residues, resistant bacteria present in the animal or human gut are also excreted with the feces (Agga et al., 2015). Because of their elevated content in organic matter and nutrients, animal feces and urine (manure) are commonly applied as nutrient amendments in soil. Such practices are appointed to contribute to the spread and dissemination of both antibiotic residues and antibiotic resistant bacteria and genes from the veterinary origin. This results in pollution of soil, crops, groundwater and adjacent surface waters (Agga et al., 2015; Chee-Sanford et al., 2009; Jechalke et al., 2014). Aquaculture is another documented source of veterinary antibiotics and antibiotic resistant bacteria pollution towards natural ecosystems, especially in low or middle-income countries where high levels of antibiotics are applied (Cabello et al., 2013).

Surface waters are also polluted with antibiotic residues and antibiotic resistant bacteria from human origin. In low- or middle-income countries, a portion of the population lacks essential sanitation infrastructures, leading to fecal residues being directly discharged into adjacent water bodies (ditches, rivers, lakes, or sea waters) without any pre-treatment (Deshpande et al., 2020). In high-income countries, fecal residues are collected in sewage systems and treated in wastewater treatment plants (WWTPs), although occasional stormwater overflows directly release antibiotics and ARGs in the environment (Eramo et al., 2017; Madoux-Humery et al., 2015). Wastewater facilities are primarily designed to remove organic and inorganic nutrients but neither pathogens nor pharmaceuticals. Arguably, WWTPs have been proposed as hotspots for the exchange and proliferation and selection of antibiotic resistant bacteria and genes due to their high bacterial densities and usual presence of selectors for antibiotic resistance genes co- and cross-selection (i.e. antibiotics, heavy metals and biocides) (Rizzo et al., 2013). Even if a fraction of both pharmaceuticals and antibiotic-resistant bacteria (and their genes) are partially removed from the waterline by the treatment, both pollutants are still discharged in higher levels than the ones present in the receiving waterbodies (Sabri et al., 2018). In addition, part of the antibiotics and bacteria that are “removed” from the waterline are sorbed into the activated sludge (biosolids line) of the WWTPs. In some countries, those biosolids (raw or after digestion) are also used as nutrient amendment in agricultural soils,

constituting a new route for environmental dissemination of both antibiotic residues and antimicrobial-resistant bacteria and genes towards the environment (Rahube et al., 2014).

The continuous discharge of both antibiotics and antibiotic-resistant bacteria is thought to increase the resistome in the receiving ecosystem (Storteboom et al., 2010). Yet the impact might vary depending on the ecosystem and other sources of ARGs pollution (Czekalski et al., 2015). Assessing the risk of such discharges for human health remains challenging (Huijbers et al., 2015). Exposure through activities in recreational waters (Leonard et al., 2018) or crops grown in soils irrigated with treated wastewater (Fahrenfeld et al., 2013) are proposed as routes for the acquisition of environmental resistance bacteria and their genes. Ultimately, data collection on the occurrence of ARGs from the discharge sources and the impacted environments is fundamental to perform the risk assessments.

1.6. Wastewater treatment

1.6.1. Origin and purpose of wastewater treatment and sanitation.

Water is a primary resource for human activities, from simple domestic use (personal care, cooking or doing laundry) to advance industrial processes (food, textile or chemical). As a result of these uses, water gets contaminated with physical, chemical, and biological pollutants that should be eliminated or, at least, reduced before the discharge towards natural water bodies (Metcalf and Eddy, 2003). Sanitation via sewage collection and treatment has been the key to safeguard public and environmental health. Ancient civilizations already applied wastewater collection by sanitation systems and even initial pre-treatment (solids sedimentation) upon discharge. However, it was not until the late 19th century and the beginning of the 20th century that water started to be treated to remove those "pollutants" using engineered settings (Lofrano and Brown, 2010). Primarily only large solids and organic nutrients were removed. The removal of nitrogen and phosphorus did not start until the 1960s, aiming to prevent eutrophication and algal blooms of the receiving water bodies, including drinking water sources (Henze et al., 2005). Recently, the presence of other chemical pollutants (micropollutants) such as pharmaceuticals (including antibiotics) or personal care products have been acknowledged as substances of emerging concern in surface water (European directive 2013/39/EEC and subsequent amendments). Yet, many of the current wastewater treatment facilities are not designed to remove those compounds. A similar situation is observed for microbiological pollution. Although the comeback of wastewater sanitation in the 17th century was boosted to face the increasing rates of infectious diseases in growing cities (Chadwick,

1834), wastewater systems and treatments were not primary designed to remove pathogens. Nevertheless, WWTPs manage to reduce a significant portion of the pathogenic bacteria and virus present in the influent (ca. 90-99%). However, unlike for nutrients removal (European directive 91/271, EEC), no general regulations for removing pathogens and discharge limits are set in the European Union (Bernasconi et al., 2003). Only the bathing water directives provide some guidance on the maximum safe concentrations of enteric pathogens in freshwaters (Directive 2006/7/EC)

1.6.2. Wastewater treatment generalities

As previously mentioned, raw wastewater can contain different contaminants that need to be removed in the subsequent treatment steps. In **Chapters 2 and 3**, the role of different stages and treatment parameters in removing ARGs is explored. Thus, a brief description of the standard treatment steps of WWTPs, relevant wastewater treatment parameters and nomenclature is provided. Such general knowledge is essential for the design of sampling campaigns, elaborating mass balances, and the global understanding of the fate of chemical and biological pollutants across wastewater treatment chains. A diagram of conventional wastewater treatment is provided in **Figure 1.5**.

1.6.3. Conventional wastewater treatment

Primary treatment consists of the removal of sand and large particles from sewage. Raw wastewater often carries sand, grit and large solids particles such as branches and wet wipes. These solids may clog and hamper the downstream water treatment processes and, therefore, must be removed within the first step in wastewater treatment. The removal of these large particles is achieved through sieves and screens, and the elimination of sand and grit is subsequently performed in the so-called grit chambers. Grit chambers are based on a quick sedimentation process, settling particles with a diameter of >0.15 mm. Large debris particles and sand often hinder the downstream processing of wastewater samples in the laboratory. Bearing in mind these technical difficulties and their quick removal upon the treatment, in this thesis, samples further referred as "**influent**" stand for wastewater already subjected to at least large solids removal, and often sand removal steps within the primary treatment.

Primary sedimentation/clarification is designed to remove undissolved wastewater particles by gravity sedimentation in large sedimentation tanks. The sedimented particles and organic matter constitute the "primary sludge". Primary clarification also allowed removing lower density substances such as oil-based compounds by scrapping the surface of the static

wastewater. In some treatment configurations, the primary sedimentation step may be skipped, and settleable particles might be stabilized directly in the biological treatment.

Secondary treatment consists of the biological treatment of wastewater. The biological treatment is the core of wastewater treatment and aims to remove the dissolved and suspended solids of wastewater, focusing on organic matter and nutrients like nitrogen and phosphorus. Many different configurations of biological treatment are nowadays available, but the majority of them (if not all) rely on the metabolic properties of microorganisms (mainly bacteria) to consume, degrade and transform the organic and inorganic compounds in wastewater. Microorganisms are commonly aggregated in flocs or biofilms, either attached as a biofilm to fixed surfaces (trickling filters) or moving carries. These microorganisms can also be in suspension forming ~ 20 µm flocs, constituting the so-called flocculent activated sludge. The majority of the wastewater treatment plants sampled in this thesis present the second configuration.

Within activated sludge systems, the flocs containing bacteria and the wastewater are intensively mixed by aeration or simple mixing, creating a gradient of redox conditions within the activated sludge basin. These varied redox conditions are necessary to carry out the different metabolic reactions for nutrient removal. Organic carbon compounds are assimilated and degraded in the aerobic zone. Simultaneously, ammonia is subsequently converted to nitrite, nitrate and finally nitrogen gas across the aerobic and anoxic zones by nitrification and denitrification, respectively. In Europe, denitrification is primarily implemented for WWTP catchment areas connected to the North Sea (sensitive to nitrate) like in the Netherlands. WWTPs from regions coupled to the Mediterranean Sea are usually only nitrifying (Weissbrodt, 2012). The total amount of oxygen that would be needed to (chemically) oxidize the organic matter is denoted as chemical oxygen demand (COD). Phosphate can be removed either chemically by adding iron or aluminium salts, or biologically by phosphate-accumulating organisms (PAOs). Selection for these organisms is achieved by the alternation of anaerobic and anoxic zones within the biological treatment.

Under dry weather conditions, wastewater will undergo several rounds of recirculation in the activated sludge basin. Afterwards, the treated wastewater will overflow to the secondary clarifier. There, sludge flocs will settle by gravity in the bottom of the clarifier, conforming to the secondary sludge. Part of the secondary sludge will be recirculated to the aeration basin, while the remaining surplus sludge will be redirected to the digester. The time that the microorganisms

spend in the biological systems is called solids retention time (SRT) or sludge age. After the activated sludge particles have settled the clean supernatant will exit the clarifier constituting the effluent that will be discharged to the adjacent natural or artificial water body (canals, rivers, lakes or sea waters).

The quality of the effluent is determined by its COD, nitrogen and phosphorus concentration. Quality criteria which must comply with the corresponding legislation (Directive 91/271/EEC). Another parameter for effluent quality is measured by the fraction of suspended particles (mainly small flocs) that remain in the effluent (Directive 91-271/EEC). These particles are referred to as total suspended solids (TSS). TSS is composed of biomass (*i.e.*, volatile suspended solids, VSS) and inorganic materials (*i.e.*, inorganic suspended solids, ISS or "ash" fraction). Antibiotic resistant bacteria and ARGs will be primarily contained in the VSS fraction.

Under dry weather conditions, wastewater spends (on average) 12-24 h to complete the treatment. This time is known as the hydraulic retention time (HRT), which is directly related to incoming wastewater and the plant design.

Tertiary and advanced treatment consists of additional treatment by *e.g.*, advanced oxidation or sorption processes to remove emerging contaminants such as organic micropollutants. In recent years, a new focus has been set on the downstream treatment of the effluent to improve its quality, particularly if this stream is meant to be used as reclaimed water (Eggen et al., 2014). These new technologies target a further removal of nutrients and suspended solids but also of pollutants that are resilient to be degraded by the conventional treatment and are a hazard for the environment (Gerba and Pepper, 2019). These resilient compounds are pharmaceuticals (including antibiotics), personal care products or bacteria (including antibiotic resistant bacteria) and viruses that survive the conventional treatment (Rosenfeld and Feng, 2011). Advanced treatment technologies aiming to eliminate these pollutants may be based on physical separation (sand filtration, coagulation). Others aim to degrade and remove these compounds by chemical reactions with ozone, UV and/or chlorination (Gerba and Pepper, 2019). However, advanced treatment technologies are not yet commonly applied in full-scale treatment plants in The Netherlands. Thus, the facilities sampled in this thesis did not feature advanced treatment steps.

Anaerobic digestion of biosolids is used to decrease the excess sludge, and simultaneously produce biogas on-site. During the various steps of wastewater treatment, a significant amount of sludge is produced (Gerba and Pepper, 2019). These biosolids contain a

high content in organics and other essential nutrients such as phosphorus and nitrogen, which lead to activated sludge being traditionally used as a soil fertilizer. However, activated sludge harbours a high concentration of volatile organics, causing a bad odour and might also contain a high abundance of pathogens (Lofrano and Brown, 2010). To reduce these problems, sludge is typically subjected to mesophilic (37ºC) anaerobic digestion. Anaerobic digestion relies on microbial anaerobic metabolism (fermentation) to degrade part of the organic matter in the absence of oxygen, generating methane as a by-product. Methane (biogas) is then used to power the treatment plant. The end product of the anaerobic digestion (digested sludge) is subsequently dewatered and dried and then used as soil fertilizer (i.e., in the UK) or incinerated (i.e. The Netherlands). If well managed, mesophilic anaerobic digestion can reduce up to 2 logs of relevant pathogens such as *Escherichia coli* or *Salmonella* spp. (Smith et al., 2005). As a result of the sludge dewatering process, reject water is produced and (in some WWTPs) recirculated to the treatment system.

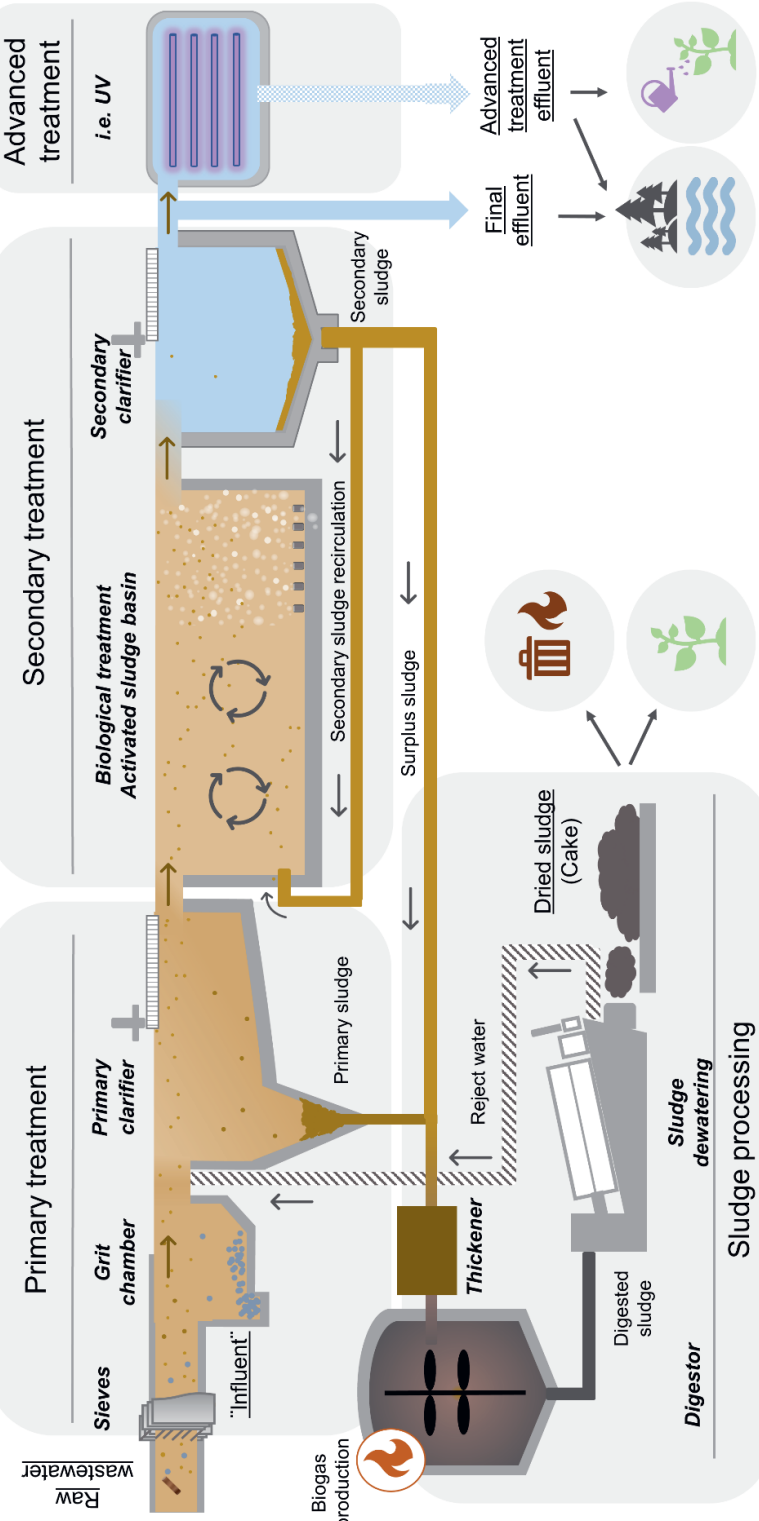


Figure 1.5. Scheme of a wastewater treatment plant process, with both water and biosolids line (sludge) featured. Facilities in the Netherlands usually lack advanced treatment, and they dispose of the dried sludge for incineration rather than crop fertilization.

1.6.4. Aerobic granular sludge-based wastewater treatment

Wastewater based on flocculent activated sludge requires extensive facilities to accommodate both the activated sludge tanks for the treatment of the wastewater and secondary clarifiers to separate the slow-settling flocculent sludge. An alternative technology that reduces this footprint by up to 75% was established over the last decade, based on aggregates displaying a dense granular shape instead of a flocculent nature (de Kreuk et al., 2007). These aggregates have a greater settling velocity than flocs, which allows a faster separation from the treated water, removing the need for secondary clarifiers and reducing the space requirements. There is also no need for separate tanks or areas within the aeration basin to perform nutrient removal.

The so-called aerobic granular sludge is operated in sequential fed-batch reactor modes, and each operational cycle consists of an anaerobic fill and effluent displacement phase, aeration phase, and settling phase operated within the same reactor (**Figure 1.6**). Besides, aerobic granules present a layered structure that generates a redox gradient within the granule (Pronk et al., 2015). In an onion layer conception, dissolved oxygen present in the bulk liquid phase is reduced from the aerobic outer layer towards the core of the granule, allowing the co-existence and simultaneous activity of microorganisms with diverse metabolic requirements: nitrifiers in the aerobic zone and denitrifying PAOs in the anoxic and anaerobic areas. As a result of both the sequential redox operation and the different microorganisms within the same granule, the consumption and removal of wastewater organic matter (COD), nitrogen, and phosphorus can be simultaneously achieved within each aerobic sludge granule (De Kreuk et al., 2005).

Since the late 00's, aerobic granular sludge treatment has been scaled up to full-scale treatments for both domestic and industrial wastewater under the trademark Nereda®. Nowadays, there are more than 65 full-scale plants around the world, one of which (in The Netherlands), was sampled and studied during **Chapter 3**.

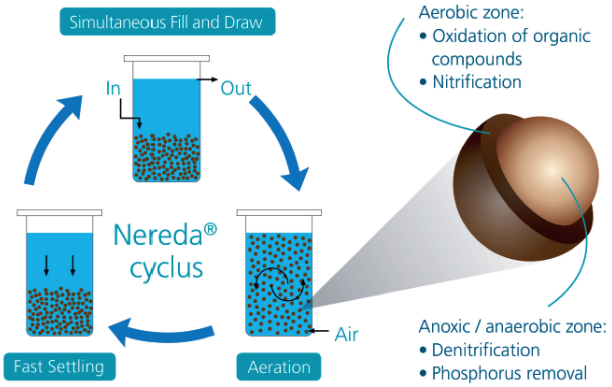


Figure 1.6. Aerobic granular sludge-based Nereda® process (left) and Redox onion-layer concept in a granule (right). Taken from Royal HaskoningDHV.

1.7. Antibiotic resistance in wastewater and wastewater treatment plants: state of the art

Even though antibiotic resistance in wastewater is known for many decades (Grabow and Prozesky, 1973), the number of published works has tremendously increased since the beginning of the century. The current research tackling antibiotic resistance in wastewater encompasses a wide variety of topics and research questions and is possible by using numerous and diverse methodologies.

1.7.1 Methods to study antibiotic resistance in wastewater

Antimicrobial residues: Antibiotic or antimicrobial residues (as well as other pharmaceuticals or chemical pollutants such as disinfectants) prevailing in the environment are usually detected employing liquid chromatography coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) (Seifrtová et al., 2009). Low concentrations in the environment, as well as matrix effects and sorption properties of different compounds, might difficult the detection of these compounds, especially in biosolids samples (Schmitt, 2017)

Culture-based methods have been fundamental to assess antibiotic resistance for many decades. Specifically, selective and differential culture media have been widely applied to enumerate, isolate and characterize different bacterial species from diverse environmental origin, including wastewater and biosolids. Culturing is likewise commonly employed to determine the antibiotic susceptibility profile of the resistant isolates, i.e determining the Minimum Inhibitory Concentration (MIC). The latter is the lower concentration of a given antibiotic inhibits bacterial growth. These analyses (used in **Chapter 4**) are performed following

guidelines from The European Committee on Antimicrobial Susceptibility Testing (EUCAST www.eucast.org), which are based on clinical isolates. Although standard epidemiological cut off resistance values (ECOFFs), based on upper limits of MICs of environmental isolates would be more appropriate, they are not commonly applied because they require a large number of isolates to be established (Karkman et al., 2018). Finally, culture-based analyses are also crucial to investigate HGT events under controlled conditions. Filter mating or mating on liquid cultures are frequently used to evaluate conjugation among recipients from the same or different species than the donors, the influence of abiotic variables on conjugation, or the effect of possible triggering factors such as antibiotics or metals.

Culture-based techniques are easy to use and relatively economical. However, there are limited by the reduced percentage of culturable bacteria, which is calculated to be only 1% of the whole bacterial kingdom (Amann et al., 1995).

Culture-independent methods overcame the culture bias and shifted the target to genetic determinants, causing resistance. Diverse molecular-based methods have become fundamental to tackle antibiotic resistance in the environment. Polymerase Chain reaction (PCR) allows the qualitative detection of genes directly from environmental DNA. PCR is also useful for the molecular profiling of antibiotic resistant isolates. Quantitative PCR (qPCR) comprised a step forward by providing accurate and sensitive quantification of molecular targets in environmental DNA, such as ARGs and MGEs. Consequently, qPCR has become the gold standard for quantification. qPCR studies are usually based on a narrow (5-10) selection of clinically relevant ARGs (covering the most relevant antibiotic families) and the MGE integrase of the integron type1, *intI1*. Integrons type 1 are common vectors carrying ARGs (Gillings et al., 2008).

A limited throughput of ARGs might provide a narrow picture of their real environmental diversity. A possible solution is qPCR-arrays, simultaneously targeting a more comprehensive range (hundreds) of genes. Yet, these methods usually provide insights about the relative quantification of genes rather than absolute abundance (i.e. gene copies per unit of volume). Other alternatives for high throughput screening are metagenomic-based techniques, potentially tracking all of described (and documented) ARGs. However, metagenomics studies often neglect the less abundant environmental ARGs because of sensitivity limitations derived from the sequencing depth (Karkman et al., 2016). Similar to qPCR arrays, metagenomics studies are useful to compare relative abundances of ARGs (semi-quantitative information) but might not be indicated to track absolute abundances. An added limitation to all PCR based

techniques (including metagenomics) is the need for previous knowledge of the molecular targets (DNA sequence) to design the appropriate primers that will detect the genes or map the reads back to the reference sequences.

Culture-independent techniques like PCR or qPCR have also been used to track conjugation in microcosms (Bellanger et al., 2014a). Moreover, recent developments such as fluorescently labelled strains and plasmids in combination with flow cytometry and cell sorting (FACS) or microscopy techniques (epifluorescence or confocal laser scanning microscopy (CLSM)) have brought additional possibilities to evaluate HGT *in situ* or under complex natural systems (Musovic et al., 2010; Seoane et al., 2011).

The disadvantages of molecular techniques are related to their considerable price (especially metagenomics) and the need for specific (expensive) equipment and trained workforce as well as bioinformatics resources. External services providing such analysis (qPCR, metagenomics) could be an alternative option to bypass some of these drawbacks.

1.7.2 Occurrence and fate of antibiotics in wastewater treatment

The concentration of antibiotics in wastewater has been analysed extensively in the last two decades (Kümmerer, 2009; Michael-Kordatou et al., 2013), particularly within the waterline. Frequently used antibiotics from varied families (aminoglycosides, β-lactams, fluoroquinolones, macrolides sulphonamides-trimethoprim and tetracyclines) have been detected in industrial and hospital effluent as well as WWTP influent and effluent (Göbel et al., 2007; Lara-Martín et al., 2014; Tahrani et al., 2016). Measured concentrations lay within the range of ng L^{-1} to a few µg L^{-1}, with pharmaceutical industry effluents and hospitals presenting the highest concentrations and the lowest in WWTP effluent (Rodriguez-Mozaz et al., 2015; Tahrani et al., 2016).

Because of their substantial prevalence in the environment and potential risk for aquatic life, three of these antibiotics (macrolides: erythromycin, clarithromycin and azithromycin) along with other pharmaceuticals were included in the EU watch list for priority substances to be monitored in water (EU Decision, 2015/495 of March 20, 2015). The list was complemented recently with the addition of amoxicillin and ciprofloxacin (EU Decision, 2018/840 of June 5, 2018), albeit only sulfamethoxazole and trimethoprim were considered in the latest update (Gomez-Cortes et al., 2020). The potential risk for aquatic life was estimated by comparing the predicted environmental concentrations (PEC) and the predicted no-effect concentrations (PNEC) for aquatic indicator microorganisms (an arthropod and two cyanobacteria (Carvalho et al., 2015)). Accordingly, these PNEC refer to ecotoxicological effects. Additional PNEC-MIC

thresholds for antibiotic resistance have been proposed (Bengtsson-Palme and Larsson, 2016). The latter would be used in **Chapter 3** of this thesis as reference thresholds for antibiotic residues in wastewater.

Besides occurrence, studies assessing temporal antibiotic fluxes or loads in wastewater have been performed (Coutu et al., 2013; Marx et al., 2015). Investigating temporal fluxes and loads is useful to reveal consumption patterns (i.e., seasonal variation), dynamics of antibiotics discharges, and effects of sampling (i.e., dilution because of rain). This knowledge is also beneficial to design more efficient and targeted sampling campaigns. Similar approaches would be appropriate for ARGs research. However, this is not the case for mass balances, commonly used in studies addressing antibiotics and the fate of other pharmaceuticals within the WWTP compartments. Unlike antibiotic residues, bacteria might grow throughout the treatment hampering a real quantification of the transfer rate through the different stages.

Mass balances of antibiotics are valuable to estimate their fate and removal rate during wastewater treatment (Lindberg et al., 2006). The removal of antimicrobials within wastewater treatment has been studied in conventional activated sludge (CAS) systems and other advanced technologies (membrane bioreactors) or additional steps (i.e., adsorption by activated carbon or degradation by advanced oxidation processes). In CAS systems, several mechanisms may remove antimicrobials. Biodegradation by activated sludge microorganisms and sorption to activated sludge are the most relevant ones (Martín et al., 2012). Some antimicrobials (i.e. tetracyclines) are mostly removed from the aqueous phase by sorption to the sludge flocs, which leads to the accumulation of these compounds within the activated sludge (Michael-Kordatou et al., 2013). Only a fraction of these antibiotics is removed by mesophilic anaerobic digestion (Lindberg et al., 2006). Thus, digested sludge and derived downstream products used as fertilizer in some countries often contain antibiotic residues in the range of mg/kg of Total Solids (TS). Unlike for the waterline, the effects of antibiotic concentrations in sludge towards macro and microorganisms (including resistant bacteria) are still poorly explored (Martín et al., 2012). This may be partially due to the rather limited data of antibiotic residues in the biosolids line of full-scale WWTP.

1.7.3 Occurrence and fate of antibiotic resistance determinants in wastewater

The presence of antibiotic resistance in raw sewage (influent) has been considered for decades. The first investigation targeted resistant isolates of the well-known coliforms (Grabow et al., 1975; Sturtevant et al., 1971) complemented later on with the inclusion of other common

fecal bacteria (i.e. Enterococci (Klare et al., 1993; Torres et al., 1994)). These early studies found resistance to diverse single antibiotics (ampicillin, kanamycin, vancomycin, among others) in varying extents (0.3-55%) and a small proportion of multi-resistant (resistant to more than three antibiotics) isolates. Further recent works have revealed a similar and even greater (20-70%) presence of antibiotic resistance (to at least 1 type of antibiotic) among the tested isolates of raw sewage (Fars et al., 2005; Ferreira Da Silva et al., 2007).

The extended occurrence of an elevated ARGs diversity in sewage has been corroborated with culture-independent methods such as qPCR arrays and metagenomics (Karkman et al., 2016; Yang et al., 2014). Unravelling the source of these resistant bacteria and genes is of interest. Hospitals have always been considered an essential source of antibiotic resistance, albeit early evidence already revealed the presence of resistant coliforms in community wastewater, although in a lower proportion than in hospital (4% and 26% respectively (Grabow and Prozesky, 1973)). Interestingly, higher percentages of resistant bacteria (ESBL *E. coli*) in hospital wastewater (13.6%) versus community (0.1-2.3%) corresponded to an equivalent absolute number of resistant isolates in both types of samples (Bréchet et al., 2014; Kwak et al., 2015). Similar results were observed for vancomycin resistant enterococci (Varela et al., 2013). When comparing influent of WWTPs with and without hospital, no significantly different levels of antibiotic resistant *E. coli* were observed (Harris et al., 2013). Since hospitals usually contribute a small fraction (ca. 1%) to the total wastewater, the impact of this compartment seems to be less relevant than initially thought (Singer et al., 2016). However, it remains a question of debate and study.

The ability of WWTPs to remove both resistant bacteria and genes during treatment is one of the fundamental questions in the topic. While some works pointed that the removal of resistant bacteria was on similar rates than total bacteria (Novo and Manaia, 2010; Vaz-Moreira et al., 2014), others observed a modest absolute reduction of antibiotic resistant enterococci (0.5-1.5 log) during treatment (Da Silva et al., 2006; Martins da Costa et al., 2006). Such sparse removals often lead to no significant reduction or even a slight increase in the relative abundance of resistant bacteria after wastewater treatment (Luczkiewicz et al., 2010). The removal of ARGs had also been explored by molecular means. By the beginning of this thesis (2016), surveillance studies were already conducted in several WTTPs across Europe (Czekalski et al., 2016; Di Cesare et al., 2016; Hembach et al., 2017; Laht et al., 2014; Rodriguez-Mozaz et al., 2015), albeit not in the Netherlands. The performance of wastewater treatment was also evaluated in other parts of the globe (Rafraf et al., 2016), especially within

Chinese facilities (Chen and Zhang, 2013; Mao et al., 2015; Wen et al., 2016). All these studies were based in a small number of plants (1-5) and targeted selected ARGs from each antibiotic group: sulfonamides (*sul1, sul2*) tetracyclines (*tetA, tetB, tetC, tetM, tetW*,) macrolides (*ermB*) quinolones (*qnrS*) and β-lactamases ($bla_{CTX\text{-}M}$, bla_{CMY}, bla_{SHV}, bla_{TEM}), as well as the integron (*intI1*). The majority informed of a decrease in the absolute concentration of ARGs after treatment, although some detected an increase in the relative abundance of some genes like bla_{TEM}, *qnrS and sul1* (Rodriguez-Mozaz et al., 2015). Some differences in the removal efficiencies were observed across WWTPs within the same studies, sometimes harbouring different treatment configurations (Di Cesare et al., 2016; Rafraf et al., 2016; Wen et al., 2016), although the causes of such discrepancies are not yet deciphered. In general, the limited amount of WWTPs in each study hindered the intra study comparison and statistical significance of possible observations. Intercomparison of studies across countries for meta-analysis remains challenging too, due to intrinsic regional differences (climate, antibiotic consumption patterns) and the lack of harmonization in the methodology (sampling, DNA extraction and qPCR methods (Manaia et al., 2015)). Despite these obstacles, relevant general insights could be drawn from the aforementioned works. For instance, which ARGs are more often in high or low occurrences in influent and effluent, and which were better or worse removed (i.e., *erm, tet >> sul, intI1*). In addition, these studies provided an average removal range for ARGs (1 to 2 log copies) after conventional wastewater treatment based on primary and secondary steps, which is useful to evaluate the success on further removal of ARGs by additional treatment steps or new treatment technologies. A few of these studies already included advance treatment steps. In some occasions, a substantial improvement in removal could be seen (Di Cesare et al., 2016) although in others the amelioration was not evident (Wen et al., 2016)

The impact of resistant bacteria and genes that are discharged with the effluent towards natural water bodies is also of great interest. An apparent increase in the percentage of resistant Enterobacteria and Aeromonas to commonly used antibiotics (tetracycline, β-lactams, co-trimoxazole) was found after the effluent discharge in a natural river in the north of Spain (Goñi-Urriza et al., 2000). An increase of either the absolute or the relative concentration of ARGs after the effluent discharges in natural water bodies has also been observed in the water fraction (Czekalski et al., 2014; Rodriguez-Mozaz et al., 2015), sediments fraction (Czekalski et al., 2014; LaPara et al., 2011) or the epilithic biofilm (Marti et al., 2013) of the receiving water bodies across Europe.

1.7.4 Antibiotic resistance in the biosolids fraction.

The biosolids fraction of wastewater treatment comprises another reservoir of antibiotic resistance genes and bacteria, although the available information is scarcer than for the waterline, particularly in full-scale treatments (Calero-Cáceres et al., 2014; Karkman et al., 2016; Munir et al., 2011; Yang et al., 2013a). From these full-scale studies, it can be concluded that there is an ample diversity of ARGs in sludge, although lower than in influent water (Karkman et al., 2016; Yang et al., 2013b; Yang et al., 2014). Besides, some specific genes (i.e. *qnrS, qnrA,* $bla_{CTX\text{-}M}$), commonly present in waterline samples and activated sludge samples, are barely detected in digested sludge (Calero-Cáceres et al., 2014).

A considerable amount of activated sludge is produced per year worldwide (i.e. 1.2 million and 5000 million kg per year in the Netherlands and China, respectively (Su et al., 2015, CBS, 2020)). Consequently, there is an imperative need to develop and improve technologies to process activated sludge in order to reuse part of the nutrients and energy contained in this product. Accordingly, a significant part of antibiotic resistance research in biosolids focused on the efficiency of diverse technologies which process the activated sludge and their impact on ARGs, primarily based on bench scale or lab-scale reactors (Diehl and LaPara, 2010; Jang et al., 2017; Ma et al., 2011; Su et al., 2015). These studies detected that anaerobic digestion caused an enrichment of particular ARGs such as tetracycline genes (varied) or erythromycin genes (*ermB*, *ermF*). Moreover, they observed that thermophilic digestion had only a slightly better performance when removing certain ARGs (Diehl and LaPara, 2010; Ma et al., 2011; Su et al., 2015). Changes in the ARGs absolute and relative abundances are thought to be a consequence of the alterations in the bacterial community throughout the anaerobic digestion processes (Ma et al., 2011)

1.7.5 Deciphering conjugation in wastewater environments.

The study of HGT events is fundamental to understand the spread of antibiotic resistance in the environment. Wastewater treatment plants are regarded as ideal ecosystems for disseminating ARGs through the horizontal exchange of plasmids (Rizzo et al., 2013).

Conjugation among different known bacterial species (i.e. gut bacteria towards environmental bacteria or *vice versa* (Goodman et al., 1993)), or even directed towards the culturable microbiota within the waterline or the biosolids line (De Gelder et al., 2005; Tsutsui et al., 2010a) has been proved first *in vitro* controlled settings (filter mating or liquid mating). These settings also contributed to determining the influence of stressors such as antibiotics

(Jutkina et al., 2016), heavy metals or biocides (Jutkina et al., 2018) within a diverse range of concentrations. From these investigations, it has been proposed that concentrations of 10 µg tetracycline, 150 times below the minimum inhibitory concentration for this antibiotic in the recipient, promoted conjugation from effluent microbiota towards *E. coli.* Other relevant abiotic factors that might affect conjugation are, for instance, nutrients or temperature, studied in **Chapter 4**.

In vitro setups are an oversimplification of the environmental systems. Consequently, more realistic settings to evaluate conjugation in the natural environment are needed. Microcosms or mesocosms (constructed ecosystems representing a simplified yet representative version of natural environments (Kangas and Adey, 1996)) have been useful to prove that conjugation can occur under environmental conditions such as water streams, soils and activated sludge (Bellanger et al., 2014b). Moreover, the development of fluorescent-tagged strains, which can be detected by culture-independent methods (FACS, CLSM) comprises a revolution in the field (Klümper et al., 2014; Sørensen et al., 2005). Still, few studies have successfully used these methods *in situ* (Seoane et al., 2011). In general, most of the works using labelled strains and FACS still rely on a culturing phase and face some relevant limitations (discussed in **Chapter 5**). Nevertheless, conjugation studies based on these techniques have been decisive to reveal the considerable diversity of environmental bacteria that can act as recipients of plasmids containing ARGs (Klümper et al., 2015).

1.8. Aims of the thesis and thesis outline

The literature addressing antibiotic resistance in wastewater is extensive and diverse, yet several questions remain unanswered and varied topics require further research. For instance, the knowledge regarding antibiotics and antibiotic resistance in Dutch wastewaters was restricted to a single work assessing the levels of resistant *E. coli* in freshwaters influenced by effluent discharges (Blaak et al., 2014). The Netherlands presents a particular and interesting study place. It comprises the European country with the lowest consumption of antibiotics (both in human and animal medicine) and holds an advanced network of WWTPs featuring diverse technologies.

In addition to the search for (somewhat) local answers, there is an awaiting need to unravel whether different technologies, plant design or operational parameters comprise a significant improvement in removing ARGs. Understanding the role of each of these components is fundamental to address possible mitigation strategies. Finally, further understanding of the effect of natural environmental conditions on the transmission of plasmids within biological systems such as activated sludge was needed.

With this in mind, the present work aimed to:

1. Unravel the occurrence of antibiotics and resistance determinants in Dutch Wastewater treatment plants throughout both their waterlines and in their biosolid lines

2. Determine the removal capacity of resistance determinants across Dutch WWTPs with conventional flocculent activated sludge treatment. Investigate whether different treatment designs and operational conditions across these treatments might favour or worsen this removal.

3. Evaluate the removal capacity of wastewater treatment systems based on aerobic granular sludge compared to conventional flocculent activated sludge.

4. Assess the influence of relevant environmental conditions present across wastewater treatment in the exchange of plasmids containing resistance determinants in controlled (filter mating) and more natural settings (microcosm).

This thesis intends to answer the questions mentioned above following a funnel approach (Figure 1.7). It starts with a general overview of Dutch wastewater treatment to specific

technologies and operational conditions and finalises looking into the transmission of plasmids under relevant environmental conditions.

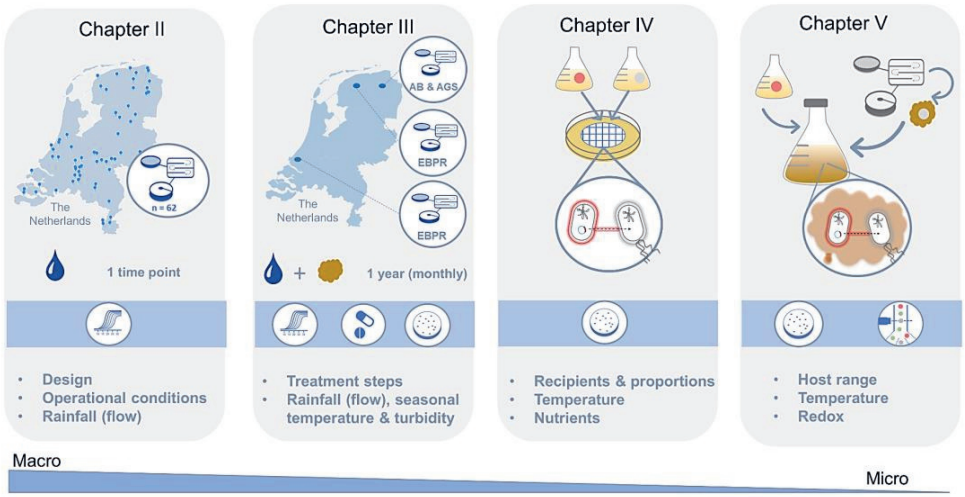


Figure 1.7. Overview of the experimental chapters of this thesis. The upper diagram depicts the workflow featured in each chapter (top). Within the blue stripe, the used techniques are highlighted, and the factors tested are displayed in bullet points.

In Chapter 2, a cross-sectional study across more than 60 WWTPs in the Netherlands evaluated antibiotic resistance. This research was performed in collaboration with the National Institute for Public Health and the Environment (RIVM). The occurrence of resistance determinants (6 ARGs and 2 MGEs) in both influent and effluent was measured. In addition, a database with information about catchment area risk factors (presence of hospitals, nursing homes), WWTPs design (capacity, type of treatment), operational parameters (HRT, SRT) and also sampling parameters (as the flow of the day of sampling) was built. This study obtained a representative picture of the current antibiotic resistance levels in Dutch wastewater systems. Moreover, we could establish the range in which these facilities (based on primary and secondary treatment steps) were able to remove the resistant determinants. Using statistical linear mixed models, the influence of these parameters in both the incoming number of genes and their effect on the removal capacity of the WWTPs was assessed. Our results provide solid proof that clinical settings do not significantly contribute to the total amount of incoming ARGs to the WWTP. More importantly, we were able to identify that the removal efficiency of the WWTPs was negatively impacted by an increase in the processed flow (presumably caused by rainfall).

Chapter 3 evaluated the occurrence and removal of resistance determinants, antimicrobials, and the fecal indicator *E. coli* in three full-scale WWTPs throughout one year. We sampled both waterline and biosolids lines across the three plants which were based on different treatment configurations (presence or lack of primary treatment or intermediate steps) and diverse biological treatment technologies (flocculent and aerobic granular sludge). Furthermore, interested in the previous chapter's outcome, we aimed to validate the influence of the flow on resistance determinants removal on a long-term study. Besides flow, we also studied the influence of other abiotic factors that were not partially addressed in the previous study, such as temperature and turbidity (as a substitute for TSS), through statistical linear mixed models.

For **Chapter 4,** we exchanged the field studies for controlled laboratory conditions. The focus also shifted to bring some answers to the conjugal transfer of antibiotic resistance related plasmids. This chapter compared *in vitro* how conjugal transfer of a broad host range plasmid (IncP-1) differed when moving from the perfect laboratory to simulated environmental conditions. Through filter mating assays, we tested the effect of donor and recipient proportions, temperature range (37º C -optimal- to 9ºC -suboptimal environmental-) and nutrient media simulating rich nutrient conditions (LB) and common environmental conditions (synthetic wastewater and soil).

Chapter 5 is a follow up of **Chapter 4**, aiming to reproduce conjugal transfer of antibiotic resistance related plasmids (broad host range IncP-1 and narrow host range IncI) under representative environmental conditions, in this case in microcosms of real activated sludge. This research was performed in collaboration with the University of Copenhagen. Besides different plasmids, two temperatures (optimal 30 and environmental 15 ºC) and two redox conditions (aerobic and anaerobic) were assessed by culture-independent and culture-dependent methods. An evaluation of the suitability of these procedures in complex biological samples is provided.

Chapter 6 provides a general discussion of the primary outcomes of this thesis and bring them into the context of the current trends in antibiotic resistance in biological treatment. This chapter also discusses the challenges encountered and provides a discussion and outlook of the future perspectives and research questions to be answered.

1.9. References

Agga, G.E., Arthur, T.M., Durso, L.M., Harhay, D.M., Schmidt, J.W., 2015. Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. PLoS One 10, 1–19. https://doi.org/10.1371/journal.pone.0132586

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., Handelsman, J., 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 8, 251–259. https://doi.org/10.1038/nrmicro2312

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169. https://doi.org/10.1128/mr.59.1.143-169.1995

Aminov, R.I., 2010. A brief history of the antibiotic era: Lessons learned and challenges for the future. Front. Microbiol. 1, 1–7. https://doi.org/10.3389/fmicb.2010.00134

Aminov, R.I., 2009. The role of antibiotics and antibiotic resistance in nature. Environ. Microbiol. 11, 2970–2988. https://doi.org/10.1111/j.1462-2920.2009.01972.x

Baltz, R.H., 2008. Renaissance in antibacterial discovery from actinomycetes. Curr. Opin. Pharmacol. 8, 557–563. https://doi.org/10.1016/j.coph.2008.04.008

Bates, S., Wilkins, B.M., 1998. IncP Plasmids Are Unusually Effective in Mediating Conjugation of. Society 180, 6538–6543.

Bellanger, X., Guilloteau, H., Bonot, S., Merlin, C., 2014a. Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review. Sci. Total Environ. 493, 872–882. https://doi.org/10.1016/j.scitotenv.2014.06.070

Bellanger, X., Guilloteau, H., Breuil, B., Merlin, C., 2014b. Natural microbial communities supporting the transfer of the IncP-1β plasmid pB10 exhibit a higher initial content of plasmids from the same incompatibility group. Front. Microbiol. 5, 1–8. https://doi.org/10.3389/fmicb.2014.00637

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ. Int. 86, 140–149. https://doi.org/10.1016/j.envint.2015.10.015

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439

Bergh, Ø., BØrsheim, K.Y., Bratbak, G., Heldal, M., 1989. High abundance of viruses found in aquatic environments. Nature 340, 467–468. https://doi.org/10.1038/340467a0

Bernasconi, C., Daverio, E., Ghiani, M., 2003. Microbiology Dimension in EU Water Directives.

Blaak, H., de Kruijf, P., Hamidjaja, R.A., van Hoek, A.H.A.M., de Roda Husman, A.M., Schets, F.M., 2014. Prevalence and characteristics of ESBL-producing E. coli in Dutch recreational waters influenced by wastewater treatment plants. Vet. Microbiol. 171, 448–459. https://doi.org/10.1016/j.vetmic.2014.03.007

Bréchet, C., Plantin, J., Sauget, M., Thouverez, M., Talon, D., Cholley, P., Guyeux, C., Hocquet, D., Bertrand, X., 2014. Wastewater treatment plants release large amounts of extended-spectrum β-lactamase-producing escherichia coli into the environment. Clin. Infect. Dis. 58, 1658–1665. https://doi.org/10.1093/cid/ciu190

Cabello, F.C., Godfrey, H.P., Tomova, A., Ivanova, L., Dölz, H., Millanao, A., Buschmann, A.H., 2013. Antimicrobial use in aquaculture re-examined: Its relevance to antimicrobial resistance and to animal and human health. Environ. Microbiol. 15, 1917–1942. https://doi.org/10.1111/1462-2920.12134

Calero-Cáceres, W., Melgarejo, A., Colomer-Lluch, M., Stoll, C., Lucena, F., Jofre, J., Muniesa, M., 2014. Sludge as a potential important source of antibiotic resistance genes in both the bacterial and bacteriophage fractions. Environ. Sci. Technol. 48, 7602–7611. https://doi.org/10.1021/es501851s

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., Møller Aarestrup, F., Hasman, H., 2014. In Silico Detection and Typing of Plasmids using PlasmidFinder and Plasmid Multilocus Sequence Typing. Antimicrob. Agents Chemother. 58, 3895–3903. https://doi.org/10.1128/AAC.02412-14

Carvalho, R.N., Ceriani, L., Ippolito, A., 2015. JCR tehnical report: Development of the first Watch List under the Environmental Quality Standards Directive water policy. https://doi.org/10.2788/101376

Cassini, A., Högberg, L.D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G.S., Colomb-Cotinat, M., Kretzschmar, M.E., Devleesschauwer, B., Cecchini, M., Ouakrim, D.A., Oliveira, T.C., Struelens, M.J., Suetens, C., Monnet, D.L., Strauss, R., Mertens, K., Struyf, T., Catry, B., Latour, K., Ivanov, I.N., Dobreva, E.G., Tambic Andraševic, A., Soprek, S., Budimir, A., Paphitou, N., Žemlicková, H., Schytte Olsen, S., Wolff Sönksen, U., Märtin, P., Ivanova, M., Lyytikäinen, O., Jalava, J., Coignard, B., Eckmanns, T., Abu Sin, M., Haller, S., Daikos, G.L., Gikas, A., Tsiodras, S., Kontopidou, F., Tóth, Á., Hajdu, Á., Guólaugsson, Ó., Kristinsson, K.G., Murchan, S., Burns, K., Pezzotti, P., Gagliotti, C., Dumpis, U., Liuimiene, A., Perrin, M., Borg, M.A., de Greeff, S.C., Monen, J.C., Koek, M.B., Elstrøm, P., Zabicka, D., Deptula, A., Hryniewicz, W., Caniça, M., Nogueira, P.J., Fernandes, P.A., Manageiro, V., Popescu, G.A., Serban, R.I., Schréterová, E., Litvová, S., Štefkovicová, M., Kolman, J., Klavs, I., Korošec, A., Aracil, B., Asensio, A., Pérez-Vázquez, M., Billström, H., Larsson, S., Reilly, J.S., Johnson, A., Hopkins, S., 2019. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect. Dis. 19, 56–66. https://doi.org/10.1016/S1473-3099(18)30605-4

Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y.-F., Yannarell, A.C., Maxwell, S., Aminov, R.I., 2009. Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genes following Land Application of Manure Waste. J. Environ. Qual. 38, 1086–1108. https://doi.org/10.2134/jeq2008.0128

Chen, H., Zhang, M., 2013. Occurrence and removal of antibiotic resistance genes in municipal wastewater and rural domestic sewage treatment systems in eastern China. Environ. Int. 55, 9–14. https://doi.org/10.1016/j.envint.2013.01.019

Colomer-Lluch, M., Jofre, J., Muniesa, M., 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. PLoS One 6. https://doi.org/10.1371/journal.pone.0017549

Cook, A.H., Lacey, M.S., 1945. Production of antibiotics by fungi. Br. J. Exp. Pathol. 26, 404–9.

Coutu, S., Wyrsch, V., Wynn, H.K., Rossi, L., Barry, D.A., 2013. Temporal dynamics of antibiotics in wastewater treatment plant influent. Sci. Total Environ. 458–460, 20–26. https://doi.org/10.1016/j.scitotenv.2013.04.017

Couturier, M., Bex, F., Bergquist, P.L., Maas, W.K., 1988. Identification and classification of bacterial plasmids. Microbiol. Rev. 52, 375–395. https://doi.org/10.1128/mmbr.52.3.375-395.1988

Cundliffe, E., 1989. How Antibiotic Producing organism avoid suicide. Annu. Rev. Genet. 43, 207–33.

Czekalski, N., Gascón Díez, E., Bürgmann, H., 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. ISME J. 8, 1381–1390. https://doi.org/10.1038/ismej.2014.8

Czekalski, N., Imminger, S., Salhi, E., Veljkovic, M., Kleffel, K., Drissner, D., Hammes, F., Bürgmann, H., Von Gunten, U., 2016. Inactivation of Antibiotic Resistant Bacteria and Resistance Genes by Ozone: From Laboratory Experiments to Full-Scale Wastewater Treatment. Environ. Sci. Technol. 50, 11862–11871. https://doi.org/10.1021/acs.est.6b02640

Czekalski, N., Sigdel, R., Birtel, J., Matthews, B., Bürgmann, H., 2015. Does human activity impact the natural antibiotic resistance background? Abundance of antibiotic resistance genes in 21 Swiss lakes. Environ. Int. 81, 45–55. https://doi.org/10.1016/j.envint.2015.04.005

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461. https://doi.org/10.1038/nature10388

Da Silva, M.F., Tiago, I., Veríssimo, A., Boaventura, R.A., Nunes, O.C., Manaia, C.M., 2006. Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. FEMS Microbiol. Ecol. 55, 322–329. https://doi.org/10.1111/j.1574-6941.2005.00032.x

Dahlberg, C., Bergström, M., Hermansson, M., 1998. In situ detection of high levels of horizontal plasmid transfer in marine bacterial communities. Appl. Environ. Microbiol. 64, 2670–2675. https://doi.org/10.1128/aem.64.7.2670-2675.1998

Datta, N., Hedges, R.W., 1971. Compatibility groups among fi - R factors. Nature 234, 222–3. https://doi.org/10.1038/234222a0

Davies, J., Davies, D., 2010. Origins and Evolution of Antibiotic Resistance. Microbiol. Mol. Biol. Rev. 74, 417–433. https://doi.org/10.1128/MMBR.00016-10

Davison, J., 1999. Genetic exchange between bacteria in the environment. Plasmid 42, 73–91. https://doi.org/10.1006/plas.1999.1421

De Gelder, L., Vandecasteele, F.P.J., Brown, C.J., Forney, L.J., Top, E.M., 2005. Plasmid donor affects host range of promiscuous IncP-1β plasmid pB10 in an activated-sludge microbial community. Appl. Environ. Microbiol. 71, 5309–5317. https://doi.org/10.1128/AEM.71.9.5309-5317.2005

de Kreuk, M.K., Heijnen, J.J., Van Loosdrecht, M.C.M., 2005. Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. Biotechnol. Bioeng. 90, 761–769. https://doi.org/10.1002/bit.20470

de Kreuk, M.K., Kishida, N., van Loosdrecht, M.C.M., 2007. Aerobic granular sludge - State of the art. Water Sci. Technol. 55, 75–81. https://doi.org/10.2166/wst.2007.244

Deshpande, A., Miller-Petrie, M.K., Lindstedt, P.A., et al., 2020. Mapping geographical inequalities in access to drinking water and sanitation facilities in low-income and middle-income countries, 2000–17. Lancet Glob. Heal. 8, e1162–e1185.

Di Cesare, A., Eckert, E.M., D'Urso, S., Bertoni, R., Gillan, D.C., Wattiez, R., Corno, G., 2016. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. Water Res. 94, 208–214. https://doi.org/10.1016/j.watres.2016.02.049

Diehl, D.L., LaPara, T.M., 2010. Effect of Temperature on the Fate of Genes Encoding Tetracycline Resistance and the Integrase of Class 1 Integrons within Anaerobic and Aerobic Digesters Treating Municipal Wastewater Solids. Environ. Sci. Technol. 44, 9128–9133. https://doi.org/10.1021/es102765a

Drake, J.W., Charlesworth, B., Charlesworth, D., Crow, J.F., 1998. Estimation of Spontaneous Mutation Rates. Genetics 1667–1686. https://doi.org/148.4.1667

Dröge, M., Pühler, A., Selbitschka, W., 2000. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. Mol. Gen. Genet. 263, 471–482. https://doi.org/10.1007/s004380051191

Du, L., Liu, W., 2012. Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. Agron. Sustain. Dev. 32, 309–327. https://doi.org/10.1007/s13593-011-0062-9

ECC, 2006. Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC. Off. J. Eur. Union L 064, 37–51.

ECDC/EMEA Joint Working Group., 2009. The bacterial challenge : time to react. https://doi.org/10.2900/2518

Eggen, R.I.L., Hollender, J., Joss, A., Schärer, M., Stamm, C., 2014. Reducing the discharge of micropollutants in the aquatic environment: The benefits of upgrading wastewater treatment plants. Environ. Sci. Technol. 48, 7683–7689. https://doi.org/10.1021/es500907n

Eramo, A., Reyes, H.D., Fahrenfeld, N.L., 2017. Partitioning of antibiotic resistance genes and fecal indicators varies intra and inter-storm during combined sewer overflows. Front. Microbiol. 8, 1–13. https://doi.org/10.3389/fmicb.2017.02024

ESAC-Net, 2017. Summary of the latest data on antibiotic consumption in the European Union. Ecdc 19, 1–13.

EU Decision, 2018. 840 of 5 June 2018 establishing a watch list of substances for Union wide monitoring in the fied of water policy. Off. J. Eur. Union 141, 9–12.

EU Decision, 2015. 495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. Off. J. Eur. Union L78/40, 20–30.

Fahrenfeld, N., Ma, Y., O'Brien, M., Pruden, A., 2013. Reclaimed water as a reservoir of antibiotic resistance genes: Distribution system and irrigation implications. Front. Microbiol. 4, 1–10. https://doi.org/10.3389/fmicb.2013.00130

Fars, S., Oufdou, K., Nejmeddine, A., Hassani, L., Melloul, A.A., Bousselhaj, K., Amahmid, O., Bouhoum, K., Lakmichi, H., Mezrioui, N., 2005. Antibiotic resistance and survival of faecal coliforms in activated sludge system in a semi-arid region (Beni Mellal, Morocco). World J. Microbiol. Biotechnol. 21, 493–500. https://doi.org/10.1007/s11274-004-2613-6

Ferreira Da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O.C., Manaia, C.M., 2007. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. FEMS Microbiol. Ecol. 60, 166–176. https://doi.org/10.1111/j.1574-6941.2006.00268.x

Fevre, C., Jbel, M., Passet, V., Weill, F.X., Grimont, P.A.D., Brisse, S., 2005. Six groups of the OXY β-lactamase evolved over millions of years in Klebsiella oxytoca. Antimicrob. Agents Chemother. 49, 3453–3462. https://doi.org/10.1128/AAC.49.8.3453-3462.2005

Galhardo, R.S., Hastings, P.J., Rosenberg, S.M., 2007. Mutation as a Stress Response and the Regulation of Evolvability. Crit. Rev. Biochem. Mol. Biol. 42, 399–435. https://doi.org/10.1080/10409230701648502

Gerba, P., Pepper, I., 2019. Environmental and Pollution Science, 3rd ed, Environmental and Pollution Science. Academic Press. https://doi.org/10.1016/C2017-0-00480-9

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., Stokes, H.W., 2008. The evolution of class 1 integrons and the rise of antibiotic resistance. J. Bacteriol. 190, 5095–5100. https://doi.org/10.1128/JB.00152-08

Göbel, A., McArdell, C.S., Joss, A., Siegrist, H., Giger, W., 2007. Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. Sci. Total Environ. 372, 361–371. https://doi.org/10.1016/j.scitotenv.2006.07.039

Goñi-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Pierre Caumette, C.Q., 2000. Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and Aeromonas spp. Appl. Environ. Microbiol. 66, 125–132. https://doi.org/10.1128/AEM.66.1.125-132.2000

Goodman, A.E., Hild, E., Marshall, K.C., Hermansson, M., 1993. Conjugative plasmid transfer between bacteria under simulated marine oligotrophic conditions. Appl. Environ. Microbiol. 59, 1035–1040. https://doi.org/10.1128/aem.59.4.1035-1040.1993

Grabow, W.O., Prozesky, O.W., 1973. Drug resistance of coliform bacteria in hospital and city sewage. Antimicrob. Agents Chemother. 3, 175–180. https://doi.org/10.1128/AAC.3.2.175

Grabow, W.O.K., Prozesky, O.W., Smith, L.S., 1975. Drug resistant coliforms call for re evaluation of water quality standards. Water Pollut. Control 74, 217–224.

Halary, S., Leigh, J.W., Cheaib, B., Lopez, P., Bapteste, E., 2010. Network analyses structure genetic diversity in independent genetic worlds. Proc. Natl. Acad. Sci. U. S. A. 107, 127–132. https://doi.org/10.1073/pnas.0908978107

Harris, S., Morris, C., Morris, D., Cormican, M., Cummins, E., 2013. The effect of hospital effluent on antimicrobial resistant E. coli within a municipal wastewater system. Environ. Sci. Process. Impacts 15, 617–622. https://doi.org/10.1039/c2em30934c

Hasegawa, H., Suzuki, E., Maeda, S., 2018. Horizontal plasmid transfer by transformation in Escherichia coli: Environmental factors and possible mechanisms. Front. Microbiol. 9, 1–6. https://doi.org/10.3389/fmicb.2018.02365

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E.T., Schwartz, T., 2017. Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. Front. Microbiol. 8, 1–11. https://doi.org/10.3389/fmicb.2017.01282

Henze, M., van Loosdrecht, M.C.M., Ekama, G.A., Brdjanovic, D., 2005. Biological wastewater treatment, Chemical Engineering.

Hernando-Amado, S., Coque, T.M., Baquero, F., Martínez, J.L., 2020. Antibiotic Resistance: Moving From Individual Health Norms to Social Norms in One Health and Global Health. Front. Microbiol. 11, 1–20. https://doi.org/10.3389/fmicb.2020.01914

Huijbers, P.M.C., Blaak, H., De Jong, M.C.M., Graat, E.A.M., Vandenbroucke-Grauls, C.M.J.E., De Roda Husman, A.M., 2015. Role of the Environment in the Transmission of Antimicrobial Resistance to Humans: A Review. Environ. Sci. Technol. 49, 11993–12004. https://doi.org/10.1021/acs.est.5b02566

Jang, H.M., Shin, J., Choi, S., Shin, S.G., Park, K.Y., Cho, J., Kim, Y.M., 2017. Fate of antibiotic resistance genes in mesophilic and thermophilic anaerobic digestion of chemically enhanced primary treatment (CEPT) sludge. Bioresour. Technol. 244, 433–444. https://doi.org/10.1016/j.biortech.2017.07.153

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., Smalla, K., 2014. Fate and effects of veterinary antibiotics in soil. Trends Microbiol. 22, 536–545. https://doi.org/10.1016/j.tim.2014.05.005

Johnston, C., Martin, B., Fichant, G., Polard, P., Claverys, J.P., 2014. Bacterial transformation: Distribution, shared mechanisms and divergent control. Nat. Rev. Microbiol. 12, 181–196. https://doi.org/10.1038/nrmicro3199

Jutkina, J., Marathe, N.P., Flach, C.F., Larsson, D.G.J., 2018. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. Sci. Total Environ. 616–617, 172–178. https://doi.org/10.1016/j.scitotenv.2017.10.312

Jutkina, J., Rutgersson, C., Flach, C.F., Joakim Larsson, D.G., 2016. An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. Sci. Total Environ. 548–549, 131–138. https://doi.org/10.1016/j.scitotenv.2016.01.044

Kangas, P., Adey, W., 1996. Mesocosms and ecological engineering. Ecol. Eng. 6, 1–5. https://doi.org/10.1016/0925-8574(95)00058-5

Kapoor, G., Saigal, S., Elongavan, A., 2017. Action and resistance mechanisms of antibiotics: A guide for clinicians. J. Anaesthesiol. Clin. Pharmacol. 33, 300. https://doi.org/10.4103/joacp.JOACP_349_15

Karkman, A., Do, T.T., Walsh, F., Virta, M.P.J., 2018. Antibiotic-Resistance Genes in Waste Water. Trends Microbiol. 26, 220–228. https://doi.org/10.1016/j.tim.2017.09.005

Karkman, A. Johnson, T.A., Lyra, C., Stedtfeld, R.D., Tamminen, M., Tiedje, J.M., Virta, M., 2016. High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. FEMS Microbiol. Ecol. 92. https://doi.org/10.1093/femsec/fiw014

Kenzaka, T., Tani, K., Nasu, M., 2010. High-frequency phage-mediated gene transfer in freshwater environments determined at single-cell level. ISME J. 4, 648–659. https://doi.org/10.1038/ismej.2009.145

Klare, I., Heier, H., Claus, H., Witte, W., 1993. Environmental strains of Enterococcus faecium with inducible high-level resistance to glycopeptides. FEMS Microbiol. Lett. 106, 23–29. https://doi.org/10.1111/j.1574-6968.1993.tb05930.x

Klümper, U., Droumpali, A., Dechesne, A., Smets, B.F., 2014. Novel assay to measure the plasmid mobilizing potential of mixed microbial communities. Front. Microbiol. 5, 1–9. https://doi.org/10.3389/fmicb.2014.00730

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945. https://doi.org/10.1038/ismej.2014.191

Kümmerer, K., 2009. Antibiotics in the aquatic environment - A review - Part I. Chemosphere 75, 417–434. https://doi.org/10.1016/j.chemosphere.2008.11.086

Kwak, Y.K., Colque, P., Byfors, S., Giske, C.G., Möllby, R., Kühn, I., 2015. Surveillance of antimicrobial resistance among Escherichia coli in wastewater in Stockholm during 1 year: Does it reflect the resistance trends in the society? Int. J. Antimicrob. Agents 45, 25–32. https://doi.org/10.1016/j.ijantimicag.2014.09.016

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., Kisand, V., 2014. Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. PLoS One 9, 1–8. https://doi.org/10.1371/journal.pone.0103705

Lapara, T.M., Burch, T.R., McNamara, P.J., Tan, D.T., Yan, M., Eichmiller, J.J., 2011. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. Environ. Sci. Technol. 45, 9543–9549. https://doi.org/10.1021/es202775r

Lara-Martín, P.A., González-Mazo, E., Petrovic, M., Barceló, D., Brownawell, B.J., 2014. Occurrence, distribution and partitioning of nonionic surfactants and pharmaceuticals in the urbanized Long Island Sound Estuary (NY). Mar. Pollut. Bull. 85, 710–719. https://doi.org/10.1016/j.marpolbul.2014.01.022

Laxminarayan, R., Van Boeckel, T., Teillant, A., 2015. The economic costs of withdrawing antimicrobial growth promoters from the livestock sector.

Lederberg, J., 1952. Cell Genetics and Hereditary Symbiosis. Physiol. Rev. 32, 403–430. https://doi.org/10.1152/physrev.1952.32.4.403

Lederberg, J., Cavalli, L.L., Lederberg, E.M., 1952. Sex Compatibility in Escherichia Coli. Genetics.

Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Hawkey, P.M., Murray, A.K., Ukoumunne, O.C., Gaze, W.H., 2018. Exposure to and colonisation by antibiotic-resistant E. coli in UK coastal water users: Environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). Environ. Int. 114, 326–333. https://doi.org/10.1016/j.envint.2017.11.003

Lindberg, R.H., Olofsson, U., Rendahl, P., Johansson, M.I., Tysklind, M., Andersson, B.A.V., 2006. Behavior of fluoroquinolones and trimethoprim during mechanical, chemical, and active sludge treatment of sewage water and digestion of sludge. Environ. Sci. Technol. 40, 1042–1048. https://doi.org/10.1021/es0516211

Lofrano, G., Brown, J., 2010. Wastewater management through the ages: A history of mankind. Sci. Total Environ. 408, 5254–5264. https://doi.org/10.1016/j.scitotenv.2010.07.062

Luczkiewicz, A., Jankowska, K., Fudala-Książek, S., Olańczuk-Neyman, K., 2010. Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. Water Res. 44, 5089–5097. https://doi.org/10.1016/j.watres.2010.08.007

Ma, Y., Wilson, C.A., Novak, J.T., Riffat, R., Aynur, S., Murthy, S., Pruden, A., 2011. Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and class i integrons. Environ. Sci. Technol. 45, 7855–7861. https://doi.org/10.1021/es200827t

Madoux-Humery, A.S., Dorner, S.M., Sauvé, S., Aboulfadl, K., Galarneau, M., Servais, P., Prévost, M., 2015. Temporal analysis of E. coli, TSS and wastewater micropollutant loads from combined sewer overflows: Implications for management. Environ. Sci. Process. Impacts 17, 965–974. https://doi.org/10.1039/c5em00093a

Manaia, C.M., Macedo, G., Fatta-Kassinos, D., Nunes, O.C., 2015. Antibiotic resistance in urban aquatic environments: can it be controlled? Appl. Microbiol. Biotechnol. 1–15. https://doi.org/10.1007/s00253-015-7202-0

Mao, D., Luo, Y., Mathieu, J., Wang, Q., Feng, L., Mu, Q., Feng, C., Alvarez, P.J.J., 2014. Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. Environ. Sci. Technol. 48, 71–78. https://doi.org/10.1021/es404280v

Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q., Alvarez, P.J.J., 2015. Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. Water Res. 85, 458–466. https://doi.org/10.1016/j.watres.2015.09.010

Marti, E., Jofre, J., Balcazar, J.L., 2013. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment tlant. PLoS One 8, 1–8. https://doi.org/10.1371/journal.pone.0078906

Martín, J., Camacho-Muñoz, D., Santos, J.L., Aparicio, I., Alonso, E., 2012. Occurrence of pharmaceutical compounds in wastewater and sludge from wastewater treatment plants: Removal and

ecotoxicological impact of wastewater discharges and sludge disposal. J. Hazard. Mater. 239–240, 40–47. https://doi.org/10.1016/j.jhazmat.2012.04.068

Martins da Costa, P., Vaz-Pires, P., Bernardo, F., 2006. Antimicrobial resistance in Enterococcus spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. Water Res. 40, 1735–1740. https://doi.org/10.1016/j.watres.2006.02.025

Marx, C., Mühlbauer, V., Schubert, S., Oertel, R., Ahnert, M., Krebs, P., Kuehn, V., 2015. Representative input load of antibiotics to WWTPs: Predictive accuracy and determination of a required sampling quantity. Water Res. 76, 19–32. https://doi.org/10.1016/j.watres.2015.02.049

Metcalf, W., Eddy, C., 2003. Metcalf and Eddy Wastewater Engineering: Treatment and Reuse, in: Wastewater Engineering: Treatment and Reuse McGraw Hill. New York, NY. https://doi.org/10.1016/0309-1708(80)90067-6

Michael-Kordatou, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. Water Res. 47, 957–995. https://doi.org/10.1016/j.watres.2012.11.027

Muela, A., Pocino, M., Arana, I., Justo, J.I., Iriberri, J., Barcina, I., 1994. Effect of growth phase and parental cell survival in river water on plasmid transfer between Escherichia coli strains. Appl. Environ. Microbiol. 60, 4273–4278. https://doi.org/10.1128/aem.60.12.4273-4278.1994

Munir, M., Wong, K., Xagoraraki, I., 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Res. 45, 681–693. https://doi.org/10.1016/j.watres.2010.08.033

Musovic, S., Dechesne, A., Sørensen, J., Smets, B.F., 2010. Novel Assay To Assess Permissiveness of a Soil Microbial Community toward Receipt of Mobile Genetic Elements. Appl. Environ. Microbiol. 76, 4813–4818. https://doi.org/10.1128/AEM.02713-09

Nethmap/Maran, 2018. Melles, DC Mouton, JW. National Institute for Public Health and the Environment. https://doi.org/10.21945/RIVM-2018-0046

Nielsen, K.M., Johnsen, P.J., Bensasson, D., Daffonchio, D., 2007. Release and persistence of extracellular DNA in the environment. Environ. Biosafety Res. 6, 37–53. https://doi.org/10.1051/ebr:2007031

Novo, A., Manaia, C.M., 2010. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. Appl. Microbiol. Biotechnol. 87, 1157–1166. https://doi.org/10.1007/s00253-010-2583-6

Ochman, H., Lawrence, J.G., Groisman, E.A., 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405, 299–304. https://doi.org/10.1038/35012500

Popa, O., Landan, G., Dagan, T., 2017. Phylogenomic networks reveal limited phylogenetic range of lateral gene transfer by transduction. ISME J. 11, 543–554. https://doi.org/10.1038/ismej.2016.116

Popowska, M., Krawczyk-Balska, A., 2013. Broad-host-range IncP-1 plasmids and their resistance potential. Front. Microbiol. 4, 1–8. https://doi.org/10.3389/fmicb.2013.00044

Pronk, M., de Kreuk, M.K., de Bruin, B., Kamminga, P., Kleerebezem, R., van Loosdrecht, M.C.M., 2015. Full scale performance of the aerobic granular sludge process for sewage treatment. Water Res. 84, 207–217. https://doi.org/10.1016/j.watres.2015.07.011

Rafraf, I.D., Lekunberri, I., Sànchez-Melsió, A., Aouni, M., Borrego, C.M., Balcázar, J.L., 2016. Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. Environ. Pollut. 219, 353–358. https://doi.org/10.1016/j.envpol.2016.10.062

Rahube, T.O., Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., Duenk, P., Lapen, D.R., Topp, E., 2014. Impact of fertilizing with raw or anaerobically digested sewage sludge on the abundance of antibiotic-resistant coliforms, antibiotic resistance genes, and pathogenic bacteria in soil and on vegetables at harvest. Appl. Environ. Microbiol. 80, 6898–6907. https://doi.org/10.1128/AEM.02389-14

Richaume, A., Smit, E., Faurie, G., van Elsas, J.D., 1992. Influence of soil type on the transfer of plasmid RP4p from Pseudomonas fluorescens to introduced recipient and to indigenous bacteria. FEMS Microbiol. Lett. 101, 281–291. https://doi.org/10.1111/j.1574-6968.1992.tb05785.x

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael-Kordatou, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242. https://doi.org/10.1016/j.watres.2014.11.021

Rosenfeld, P.E., Feng, L.G.H., 2011. Emerging Contaminants. Risks Hazard. Wastes 215–222. https://doi.org/10.1016/b978-1-4377-7842-7.00016-7

Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Guerra, B., Mevius, D.J., Hordijk, J., 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J. Antimicrob. Chemother. 73, 1121–1137. https://doi.org/10.1093/jac/dkx488

Sabri, N.A., Schmitt, H., Van der Zaan, B., Gerritsen, H., Zuidema, T., Rijnaarts, H.H.., Langenhoff, A.A.M., 2018. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. J. Environ. Chem. Eng. https://doi.org/10.1016/j.jece.2018.03.004

Sartelli, M., Hardcastle, T.C., Catena, F., Chichom-Mefire, A., Coccolini, F., Dhingra, S., Haque, M., Hodonou, A., Iskandar, K., Labricciosa, F.M., Marmorale, C., Sall, I., Pagani, L., 2020. Antibiotic use in low and middle-income countries and the challenges of antimicrobial resistance in surgery. Antibiotics 9, 1–12. https://doi.org/10.3390/antibiotics9080497

Schmitt, H., 2017. Bronnen va antibioticaresistentie in het milieu en mogelijke maatregelen. https://doi.org/10.21945/RIVM-2017-0058

Seifrtová, M., Nováková, L., Lino, C., Pena, A., Solich, P., 2009. An overview of analytical methodologies for the determination of antibiotics in environmental waters. Anal. Chim. Acta 649, 158–179. https://doi.org/10.1016/j.aca.2009.07.031

Seoane, J., Yankelevich, T., Dechesne, A., Merkey, B., Sternberg, C., Smets, B.F., 2011. An individual-based approach to explain plasmid invasion in bacterial populations. FEMS Microbiol. Ecol. 75, 17–27. https://doi.org/10.1111/j.1574-6941.2010.00994.x

Shallcross, L.J., Davies, D.S.C., 2014. Antibiotic overuse: a key driver of antimicrobial resistance. Br. J. Gen. Pract. 64, 604–605. https://doi.org/10.3399/bjgp14X682561

Singer, A.C., Shaw, H., Rhodes, V., Hart, A., 2016. Review of antimicrobial resistance in the environment and its relevance to environmental regulators. Front. Microbiol. 7, 1–22. https://doi.org/10.3389/fmicb.2016.01728

Smillie, C., Garcillán-Barcia, M.P., Francia, M.V., Rocha, E.P.C., de la Cruz, F., 2010. Mobility of plasmids. Microbiol. Mol. Biol. Rev. 74, 434–52. https://doi.org/10.1128/MMBR.00020-10

Smith, S.R., Lang, N.L., Cheung, K.H.M., Spanoudaki, K., 2005. Factors controlling pathogen destruction during anaerobic digestion of biowastes. Waste Manag. 25, 417–425. https://doi.org/10.1016/j.wasman.2005.02.010

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., 2005. Studying plasmid horizontal transfer in situ: a critical review. Nat. Rev. Microbiol. 3, 700–710. https://doi.org/10.1038/nrmicro1232

Storteboom, H., Arabi, M., Davis, J.G., Crimi, B., Pruden, A., 2010. Identification of Antibiotic-Resistance-Gene Molecular Signatures Suitable as Tracers of Pristine River, Urban, and Agricultural Sources. Environ. Sci. Technol. 44, 1947–1953. https://doi.org/10.1021/es902893f

Sturtevant, A.B., Cassell, G.H., Feary, T.W., 1971. Incidence of infectious drug resistance among fecal coliforms isolated from raw sewage. Appl. Microbiol. 21, 487–491. https://doi.org/10.1128/aem.21.3.487-491.1971

Su, J.Q., Wei, B., Ou-Yang, W.Y., Huang, F.Y., Zhao, Y., Xu, H.J., Zhu, Y.G., 2015. Antibiotic Resistome and Its Association with Bacterial Communities during Sewage Sludge Composting, Environmental Science and Technology. https://doi.org/10.1021/acs.est.5b01012

Suzuki, H., Yano, H., Brown, C.J., Top, E.M., 2010. Predicting plasmid promiscuity based on genomic signature. J. Bacteriol. 192, 6045–6055. https://doi.org/10.1128/JB.00277-10

Tahrani, L., Van Loco, J., Mansour, H. Ben, Reyns, T., 2016. Occurrence of antibiotics in pharmaceutical industrial wastewater, wastewater treatment plant and sea waters in Tunisia. J. Water Health 14, 208–213. https://doi.org/10.2166/wh.2015.224

Teuber, M., 2001. Veterinary use and antibiotic resistance. Curr. Opin. Microbiol. 4, 493–499. https://doi.org/10.1016/S1369-5274(00)00241-1

Thomas, C.M., Smith, C.A., 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. 41, 77–101. https://doi.org/10.1146/annurev.mi.41.100187.000453

Torres, C., Reguera, J.A., Sanmartin, M.J., Pérez-díaz, M.C., Baquero, F., 1994. Van a-mediated vancomycin-resistant enterococcus spp. in sewage. J. Antimicrob. Chemother. 33, 553–561. https://doi.org/10.1093/jac/33.3.553

Tsutsui, H., Anami, Y., Matsuda, M., Inoue, D., Sei, K., Soda, S., Ike, M., 2010. Transfer of plasmid pJP4 from Escherichia coli and Pseudomonas putida to bacteria in activated sludge developed under different sludge retention times. J. Biosci. Bioeng. 110, 684–689. https://doi.org/10.1016/j.jbiosc.2010.07.009

Varela, A.R., Ferro, G., Vredenburg, J., Yanik, M., Vieira, L., Rizzo, L., Lameiras, C., Manaia, C.M., 2013. Vancomycin resistant enterococci: From the hospital effluent to the urban wastewater treatment plant. Sci. Total Environ. 450–451, 155–161. https://doi.org/10.1016/j.scitotenv.2013.02.015

Vaz-Moreira, I., Nunes, O.C., Manaia, C.M., 2014. Bacterial diversity and antibiotic resistance in water habitats: Searching the links with the human microbiome. FEMS Microbiol. Rev. https://doi.org/10.1111/1574-6976.12062

Weber, T., Welzel, K., Pelzer, S., Vente, A., Wohlleben, W., 2003. Exploiting the genetic potential of polyketide producing streptomycetes. J. Biotechnol. 106, 221–232. https://doi.org/10.1016/j.jbiotec.2003.08.004

Weissbrodt, D.G., 2012. Bacterial Resource Management for Nutrient Removal in Aerobic Granular Sludge Wastewater Treatment Systems 5641, 423.

Wen, Q., Yang, L., Duan, R., Chen, Z., 2016. Monitoring and evaluation of antibiotic resistance genes in four municipal wastewater treatment plants in Harbin, Northeast China. Environ. Pollut. 212, 34–40. https://doi.org/10.1016/j.envpol.2016.01.043

Woegerbauer, M., Bellanger, X., Merlin, C., 2020. Cell-Free DNA: An Underestimated Source of Antibiotic Resistance Gene Dissemination at the Interface Between Human Activities and Downstream Environments in the Context of Wastewater Reuse. Front. Microbiol. 11, 1–11. https://doi.org/10.3389/fmicb.2020.00671

Wright, G.D., 2007. The antibiotic resistome: The nexus of chemical and genetic diversity. Nat. Rev. Microbiol. 5, 175–186. https://doi.org/10.1038/nrmicro1614

Xu, L., Meng, W., Cao, C., Wang, J., Shan, W., Wang, Q., 2015. Antibacterial and antifungal compounds from marine fungi. Mar. Drugs 13, 3479–3513. https://doi.org/10.3390/md13063479

Yang, Y., Li, B., Ju, F., Zhang, T., 2013a. Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach. Environ. Sci. Technol. 47, 10197–10205. https://doi.org/10.1021/es4017365

Yang, Y., Li, B., Ju, F., Zhang, T., 2013b. Exploring Variation of Antibiotic Resistance Genes in ActivatedSludge over a Four-Year Period through a Metageno.

Yang, Y., Li, B., Zou, S., Fang, H.H.P., Zhang, T., 2014. Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. Water Res. 62, 97–106. https://doi.org/10.1016/j.watres.2014.05.019

CHAPTER 2

2

Determinants of presence and removal of antibiotic resistance genes during WWTP treatment: a cross-sectional study

Abstract

Wastewater treatment plants (WWTPs), linking human fecal residues and the environment, are considered hotspots for the spread of antibiotic resistance. In order to evaluate the role of WWTPs and underlying operational parameters for the removal of resistance determinants, the presence and removal efficiency of a selected set of 6 antimicrobial resistance genes (ARGs) and 2 mobile genetic elements (MGEs) was evaluated by means of qPCR in influent and effluent samples from 62 Dutch WWTPs. The role of possible factors impacting the concentrations of ARGs and MGEs in the influent and their removal was identified through statistical analysis. ARGs and the class I integron-integrase gene (*intI1*) were, on average, removed to a similar extent (1.76 log reduction) or better (+0.30-1.90 logs) than the total bacteria (measured as 16S rRNA gene). In contrast, broad-host-range plasmids (IncP-1) had a significantly increased ($p<0.001$) relative abundance after treatment. The presence of healthcare institutions in the area served did only slightly increase the concentrations of ARGs or MGEs in influent. From the extended panel of operational parameters, rainfall, increasing the hydraulic load of the plant, most significantly ($p <0.05$) affected the treatment efficiency by decreasing it on average -0.38 logs per time the flow exceeded the average daily flow. Our results suggest that overall WWTP treatments do not favour the proliferation of the assessed resistance genes but might increase the relative abundance of broad host range plasmids of the IncP-1 type.

A modified version of this chapter has been published as: Pallares-Vega, R., Blaak, H., van der Plaats, R., de Roda Husman, A.M., Hernandez Leal, L., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2019. Determinants of presence and removal of antibiotic resistance genes during WWTP treatment: A cross-sectional study. Water Res. 161, 319–328. https://doi.org/10.1016/j.watres.2019.05.100

Acronyms and abbreviations

ADF:	Average Daily Flow
ACT:	Anaerobic Contact Time
AIC:	Akaike Information Criterion
ARG:	Antibiotic-Resistant Gene
ARB:	Antibiotic-Resistant Bacteria
B	Biological (Phosphorus removal)
BC	Biochemical (Phosphorus removal)
BOD:	Biological Oxygen Demand
C:	Chemical (Phosphorus removal)
COD:	Chemical Oxygen Demand
DF:	Daily Flow
DWF:	Dry Weather Flow
HGT:	Horizontal Gene Transfer
HRT:	Hydraulic Retention Time
MGE:	Mobile Genetic Element
N:	Nitrogen
NP:	No Primary sedimentation
P:	Phosphorus
PE:	Population Equivalents
PR:	Primary sedimentation with recirculation of reject water
PNR:	Primary sedimentation without recirculation of reject water
TOC:	Total Organic Carbon
TOD:	Total Oxygen Demand
TSS:	Total Suspended Solids
SRT:	Sludge Retention Time
UV:	Ultra Violet light
WWTP:	Wastewater Treatment Plant

2.1. Introduction

Antibiotic resistance is a growing problem worldwide. Although it is a natural and ancient phenomenon (D'Costa et al., 2011), its occurrence in natural environments has been accelerated by anthropogenic activities. One of the essential vectors for the dissemination of human-related resistance determinants into the environment is wastewater, as it collects fecal residues, including antibiotic-resistant bacteria (ARB) and their genes (ARGs) (Baquero et al., 2008).

In Europe, wastewater is treated in wastewater treatment plants (WWTPs), and their effluents are commonly discharged into natural water bodies. The main goal of sewage treatment is to remove organic components (measured as chemical (COD) and biological (BOD) oxygen demand), phosphorus and nitrogen nutrients (P, N) as well as suspended solids, but not bacteria or their genes (Council of the European Communities, 1991).

The core biological secondary treatment units of WWTPs, involving activated sludge, are composed of open microbiomes involving complex networks of microbial populations (Weissbrodt et al., 2014). The microbial communities of WWTPs are considered hotspots for horizontal gene transfer (HGT) and putative proliferation of antibiotic resistance (Berendonk et al., 2015; Rizzo et al., 2013).

Therefore, in the last years, several studies in different countries have evaluated the fate of ARGs in full-scale WWTPs (Czekalski et al., 2012; Makowska et al., 2016; Rafraf et al., 2016; Rodriguez-Mozaz et al., 2015). Whereas most studies have detected lower absolute concentrations of ARGs after treatment, inconsistent results have been found on changes in the concentration of ARG relative to 16S rRNA ("relative abundance") (Lee et al., 2017; Makowska et al., 2016; Rafraf et al., 2016). Many factors might be the cause of this disparity, including changes in community composition along with the treatment, the presence antibiotic resistance selective agents in the wastewater, as well as the sampling design. Moreover, up to date, studies have rarely investigated the relationship between the efficiency of ARG removal and the process design and operational conditions of WWTPs. One approach has been to consider possible relations between the presence or removal of ARGs and water quality parameters as temperature, total organic carbon (TOC), BOD, nutrients and TSS (Ben et al., 2017; Di Cesare et al., 2016; Laht et al., 2014; Novo et al., 2013). Even though some correlations were found (e.g., between temperature and sulphonamide ARB, or between TOC and *ermB*, *tetA* and *qnrS* ARGs), these results did not universally apply to all investigated ARG and are based on a low

number of investigated plants. Regarding the operation of the plant, most of the recent studies focus on assessing or comparing the efficiency of advanced treatments or disinfection technologies such as biological post filtration or disinfection by chlorination, UV or peracetic acid (Di Cesare et al., 2016; Laht et al., 2014).

In order to limit emissions of ARG to surface water, information on the efficiency of WWTP treatment and the role of plant processes is needed. Therefore, the aim of our study was 1) to evaluate the presence and removal of ARGs and MGEs in Dutch WWTPs and investigate changes in the relative gene abundance, and to elucidate 2) the influence of catchment area factors on loads of ARGs and MGEs in raw wastewater as well as 3) the role of WWTP process configurations for their removal. A selected panel of 6 relevant ARGs (Berendonk et al. 2015) and 2 mobile genetic elements (MGEs) related to antibiotic resistance, *intI1* and *korB* (IncP-1 plasmids), were analyzed across an extended number of 62 treatment plants. Moreover, information regarding the presence of possible explanatory variables in the catchment area *(i.e.*, healthcare institutions), the amount of treated water during the sampling day (hydraulic load), and the design configuration and operational parameters of the WWTPs were gathered. The correlation of process and/or catchment parameters with ARG abundance and removals was studied with linear mixed models.

2.2. Materials and methods

2.2.1. Characteristics of the selected Wastewater Treatment Plants and sample procedure

Influent and effluent samples were collected in 62 Dutch WWTPs distributed across the country (**Figure S2.1 in the supplementary information**). The selected plants comprised varied sizes (4800 – 1060500 Population Equivalents (pe) based on 150g TOD) and different process configurations (**Table S2.1 in the supplementary information**). No installation with advanced treatment was included. The sampling was performed at a single time point per plant within the period dating from April to November of 2016. Detailed information about the sampling points and sampling procedures can be found in Schmitt (2017). Briefly, 24-h flow-proportional composite samples were gathered from the influent and the effluent of the WWTPs. Samples were processed within 24 h upon collection, as follows: a total of 30 mL of influent and 250 mL of effluent were filtered through 0.45-µm ester-cellulose membranes (Merk Millipore, DE) and filters were frozen at -20°C until extraction.

2.2.2. DNA extraction, quantification of ARGs and MGEs by quantitative polymerase chain reaction (qPCR) and correlation analysis

DNA filters were extracted with the DNeasy kit Power Water (Qiagen, NL) following the manufacturer's instructions. DNA quantification and purification were determined by fluorometry using Qubit® (Thermofisher, US).

The diluted DNA was subjected to quantitative polymerase chain reaction (qPCR) analysis of the selected genes panel. The 16S rRNA gene was selected as a proxy to quantify total bacteria. qPCR ARGs targets were chosen from a proposed target panel to track ARGs (Berendonk et al., 2015). The chosen ARGs confer resistance to the antibiotics with the highest consumption in The Netherlands, namely: macrolides (*ermB),* tetracyclines *(tetM),* sulphonamides *(sul1* and *sul2),* fluoroquinolones *(qnrS)* and extended-spectrum beta-lactamases (*bla*$_{ctxM}$) (**Table 2.1**). Moreover, two genes assessing the presence of MGEs were included in the study: *intI1 and korB*. The first target allows detecting integrase of Integron class I, a well-known MGE related to the acquisition and exchange of ARGs through HGT events. *intI1* may also act as a marker for anthropogenic pollution (Gillings et al., 2015). The second, *korB* gene, targets plasmids belonging to the incompatibility (Inc) group IncP-1 (Jechalke et al., 2013) that are another kind of MGEs associated with ARGS and serve as models to study HGT events in complex environmental samples (Klümper et al., 2015; Tsutsui et al., 2010). Further information about oligonucleotides, probes, reaction mix, and conditions can be found in the **supplementary information and Table S2.**

Table 2.1 Panel of genes used in this study. Genes are arranged within three groups of interest: all bacteria, antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs).

Group	Gene	Function
All bacteria	16S rRNA	For normalization to the concentration of bacteria
ARGs	*ermB*	Resistance to macrolides
	sul1	Resistance to sulphonamides
	sul2	Resistance to sulphonamides
	tetM	Resistance to tetracyclines
	qnrS	Resistance to quinolones
	bla$_{CTXM}$	Resistance to extended-spectrum β-lactams
MGEs	*intI1*	Integrase of type 1 integrons (clinical and environmental)
	korB	Broad host range plasmids of incompatibility group IncP-1

2.2.3. Catchment area characteristics and plant parameters for statistical analysis

Information about healthcare institutions was obtained from a separate project (Schmitt, 2017). In brief, information on localization of hospitals and polyclinics was obtained from a registry of Dutch health data maintained by the National Institute for Public Health and the Environment (www.volksgezondheidenzorg.info) and amended with a separate list of Dutch hospitals (https://nl.wikipedia.org/wiki/Lijst_van_Nederlandse_ziekenhuizen). Information on care homes was obtained from a registry of Dutch health care institutions maintained by the Dutch patient federation. (https://www.zorgkaartnederland.nl/overzicht/sectoren/verpleeghuizen-en-verzorgingshuizen/zorgaanbieders/plaatsen). Both were matched with the areas served by specific WWTPs. The Daily Flow of the WWTPs on the sampling days was obtained from the corresponding waterboards or WWTPs. Detailed information about plant design and performance parameters for the WWTPs in 2016 was obtained through the Dutch Statistical Office (Central Bureau Statistiek –CBS, 2018 https://www.cbs.nl/en-gb). The list of parameters taken into account for statistical analysis is summarized in **Table 2.2.**

2.2.4. Statistical analyses

Statistical analyses were performed with R 3.4.4 (R Core Team, 2018) and Rstudio (https://www.rstudio.com/). Correlation analysis (Pearson's correlation) between the removal of ARGs, MGEs, and fecal indicator bacteria *Escherichia coli* were performed with the package corrplot (Wei and Simko, 2016). The data regarding *E. coli* removal was obtained from a previous study (Schmitt, 2017).

For the analysis of the relation of the absolute resistance gene concentrations in the WWTP's influent to catchment area factors, the log-transformed absolute concentration of 8 genes per litre of influent was set as the outcome variable. $Bla_{CTX\text{-}M}$ was excluded from the statistical analysis as the data set was not complete (this gene was found below the detection limit in >10% of the WWTPs effluent samples). Explanatory variables tested included the presence of hospitals, polyclinics and nursing homes in the catchment area, as well as the effect of rainfall events increasing the hydraulic load (amount of water processed) of the WWTP during the sampling day. We named this variable "Hydraulic Load Factor," and it was calculated as the ratio of the Daily Flow (DF) during the sampling day over the average daily flow (derived from the annual flow) and expressed as "times x ADF" **Eq. (2.1)**. We used the annual flow as the dry weather flow (DWF) was not known for all plants. The Hydraulic Load Factors observed in the

62 studied WWTP fall well within the distribution of Hydraulic Load Factors retrieved from daily flows of two exemplary individual WWTP over two years and are therefore representative for Dutch conditions (data not shown). A linear mixed model involving the R packages lme4 and lmerTest (to determine p values through Satterthwaite approximation) (Bates et al., 2016; Kuznetsova et al., 2017) was used with the genes and the Hydraulic Load Factor and area parameters as fixed factors and a random intercept for the plant identity. Parameters were first tested univariably, including testing for interaction between gene identity and the other factors. Then, the MuMin package (Barton, 2018) was used for the identification of the best minimum adequate models by fitting all possible submodels using maximum likelihood methods and ranking them by the corrected Akaike information criterion (AICc), retaining all models differing from the best model by less than an AICc of 2. Demonstration of single best models was chosen instead of model averaging (Dormann et al., 2018). The quality of the model was investigated by visually inspecting the normality of the residuals.

For the analysis of the influence of plant parameters on the removal efficiency, the outcome variable was the log-transformed removal efficiency per gene per plant (i.e., the log reduction). Variables tested included the following: size of the plant (based on 150 g TOD population-equivalents), presence/absence of primary sedimentation in combination with recirculation of reject water, type of P removal (none, biological, or biological and chemical), average sludge retention time (SRT), average hydraulic retention time (HRT), anaerobic contact time, and the average concentration of total suspended solids (TSS) in the effluent. As denitrification was applied in all plants, it was not included in the statistical analysis. A linear mixed model was used, with the resistance gene and the plant parameters as fixed factors and a random intercept for the plant identity. In the first step, all parameters were tested in bivariate models (gene and plant parameter), and the significance of the interaction between genes and parameters was determined. To adjust for the Hydraulic Load Factor effect, which was found to be highly significant, trivariate models were run (including the following three explanatory factors: gene, Hydraulic Load Factor, and their interaction, in addition to one additional parameter). Finally, all parameters (gene, Hydraulic Load Factor, and their interaction, the presence of primary settler with and without recirculation of reject water, type of P removal, HRT, SRT, and TSS) were tested in a full model. The anaerobic contact time was excluded from this model since data on the design of this parameter was only available for 11 out of the 62 WWTPs. The MuMin package (Barton, 2018) was used for model reduction (i.e., identification of the best submodels of this model) using maximum likelihood methods, choosing the models

differing from the best model by less than 2 AICc. The model reduction was performed on a subset of 37 plants with complete observations. The identified minimum adequate submodels were then re-run for the largest set of WWTP for which all relevant data was available. The quality of the model was investigated through analysis of the normality of the residuals. Collinearity amongst explanatory variables was assessed by variance inflation factors.

Table 2.2. Studied variables and operational parameters that possibly affect ARGs and MGEs loads and removal efficiency. Information gathered from the WWTPs, the waterboards, and the Dutch Statistical Office (CBS, 2018). The Average Daily Flow was calculated as the total annual flow divided by 365 days. Acronyms: PE: population equivalents.

Origin		Parameter
Catchment area		Presence/ absence of hospitals Presence/absence of policlinics Presence/absence of nursing homes
WWTP	Plant general information	Size of the plant in PE (150 g TOD)
		Hydraulic Retention Time (HRT) in hours (year average)
	Primary treatment	Presence/absence of primary sedimentation with and without recirculation of reject water: NP: no primary sedimentation PR: primary sedimentation with recirculation reject water PNR: primary sedimentation without recirculation of reject water
	Secondary treatment	Type of P removal: None B: Biological C: Chemical BC: Biochemical
		Sludge Retention Time (SRT) in hours (year average) Anaerobic contact time in hours (as from plant design)
	Effluent	Effluent TSS (year average) in mg/L
Sampling		Hydraulic Load Factor (as a surrogate from Rainfall) **Eq. (2.1):** $\text{HLF} = \frac{Daily\ Flow}{Average\ Daily\ Flow}$

2.3. Results and discussion

2.3.1. Prevalence of ARGs and MGEs in the influent and role of the catchment area

We assessed the occurrence of different ARGs and MGEs in the influent of 62 WWTP. Our results, summarized in **Figure 2.1** and **Table S2.3 in the supplementary information**, showed that the most predominant genes in the influent were the ARG *sul1*, *ermB* and the MGE *intI1* (6.54 log copies mL^{-1} on average). The concentrations of *tetM*, *sul2*, *qnrS,* and MGE *korB,* were on average 10 times lower, while the lowest concentrations (4.40 log copies mL^{-1}) were

obtained for the beta-lactamase gene *bla*$_{ctxM}$. These findings are in general accordance with the concentrations found in other European studies by Czekalski et al. (2014), Rodriguez-Mozaz et al. (2014) and Di Cesare et al. (2016), but 2-3 logs lower than the ones found in other northern European countries (Laht et al., 2014) (**Table S2.3**). The resistance to sulfonamides (*sul1, sul2*) and macrolides (*ermB*) seem to be most widespread, although the use of these antibiotics in humans is not so extensive anymore (10% of the total antibiotic consumption in humans in 2016 (Nethmap/Maran, 2018)). Their high prevalence may be the result of the combination of prolonged use of these antibiotics in both humans and animal husbandry, their association with MGEs (Baran et al., 2011; Davies and Davies, 2010) and the presence of residues from these antibiotic families in wastewater (Schmitt et al., 2016).

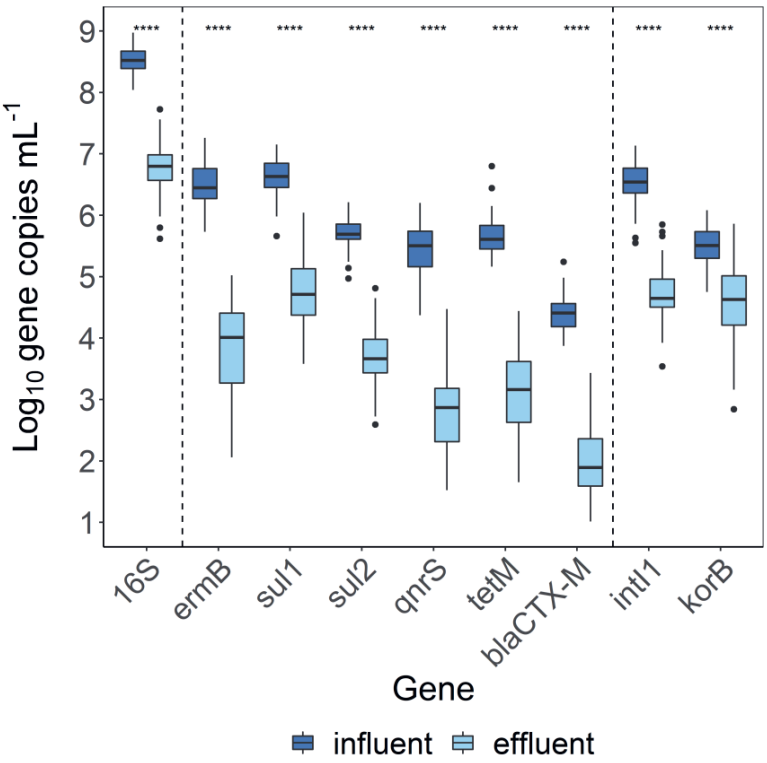


Figure 2.1. Absolute concentration of the 16S rRNA gene, ARGs, and MGEs in influent (dark blue) and effluent (light blue) samples from 62 Dutch WWTPs. Different types of genes (16S, ARGs, and MGEs) are separated by vertical lines. The results are expressed in $\log_{10}$ copies per mL^{-1}. The boxes represent the 2nd and 3rd quartiles. The middle black line represents the median, and the whiskers represent the 1st and 4th quartile. Spare black dots represent outlier values. Significant differences in gene presence after treatment were assessed by a paired Wilcoxon test, and values are indicated above each gene (****): $p<0.0001$.

The possible contribution of healthcare institutions to the ARGs and MGEs levels in influent of WWTPs was also investigated in this study. We found a small effect of the presence

of hospitals on concentrations of resistance genes in influent: while the presence of hospitals was included in all best models indicating a possible role of hospitals as point sources of resistance, the increase in gene concentrations in influents due to presence of hospital wastewater was relatively small (<0.10 log unit), and hospital presence was not significant within these models. The effects of the presence of care homes and polyclinics were yet lower (**Table 2.3 and Figure S2.2 in the supplementary information**). The role of healthcare institutions as an important source of antibiotic resistance emissions (bacteria and genes) into the sewer system has been already demonstrated in previous studies (Buelow et al., 2018; Lorenzo et al., 2018; Rodriguez-Mozaz et al., 2015). However, despite elevated concentrations of ARGs in healthcare sewage systems as compared to community or industry wastewater, the discharges from healthcare institutions are estimated to represent just 1% of the total influent volume (Kümmerer, 2009b; Schmitt, 2017). Thus, the contribution of healthcare institutions wastewater was likely too small to increase the overall concentration of the genes tested in WWTP influents. Similar conclusions were recently drawn by Buelow et al. (2018). Yet, hospitals and healthcare institutions should still be regarded as source multi-resistant strains (Verburg et al., 2019) and novel genetic combinations of interest (Sheppard et al., 2016).

Table 2.3. Effect of operational parameters and catchment factors on concentrations of ARGs and MGEs in the influent. Estimates of the effects of the explanatory variables on gene concentrations (in $\log_{10}$ copies L^{-1}) are given (with their p values between brackets and in italic– significant estimates are shown in bold) for the 6 best models that were of nearly identical quality as determined by AICc. Gene identity was also included in all models. For interaction terms, the genes for which significant interactions with explanatory variables were found are listed (for *korB* the increase of concentrations in influent with hospitals was higher than for *16S*; for *qnrS* the increase of concentrations in influents with care homes was lower than for *16S*). Acronym: n: number of plants with available information for that parameter.

Intercept	Hydraulic Load Factor	Presence of hospital	Presence of polyclinic	Presence of care home	Hospital : gene inter-action	Care home : gene inter-action	AICc	n
11.56	-0.11 *(p=0.11)*	0.08 *(p=0.34)*			*korB*		103.2	62
11.48		0.06 *(p=0.43)*			*korB*		103.7	62
11.56	-0.12 *(p=0.09)*	0.07 *(p=0.37)*		0.01 *(p=0.91)*	*korB*	*qnrS*	103.8	62
11.56	-0.12 *(p=0.09)*	0.06 *(p=0.44)*		0.07 *(p=0.35)*	*korB*		104.4	62
11.63	-0.13 *(p=0.08)*	0.10 *(p=0.24)*	-0.09 *(p=0.40)*		*korB*		104.6	62
11.48		0.06 *(p=0.43)*		-0.01 *(p=0.97)*	*korB*	*qnrS*	104.7	62

Rainfall occurring on the sampling date, increasing the Hydraulic Load Factor, was found to slightly decrease the concentration of ARGs in the influent. This was likely a consequence of diluting human waste in the sewer with the rainfall inflow (Metcalf and Eddy, 2003). Still, the effect of rainfall on the gene prevalence (-0.11 logs / per time ADF was doubled) was not significant by itself (**Table 2.3 and Figure S2.3 in the supplementary information**). These results could be explained by the homogenizing effect of the 24-h composite samples that would include wastewater from both rain showers but also the dry period. Such an effect was also observed by Lucas et al. (2014) while monitoring fecal indicator bacteria in two WWTPs in Paris. Besides, the variability in both catchment area characteristics (size, residence time within the sewer) and the rainfall events could also have influenced the magnitude of the effect as was previously observed for fecal indicators and ARGs in influent and combine sewers overflows (Eramo et al., 2017; Lucas et al., 2014). Unfortunately, information about the parameters above was not available or not possible to integrate into this study and thus, we cannot conclude their impact.

2.3.2. ARGs and MGE removal efficiencies

In the WWTPs studied, the average removal of ARGs was similar or higher than the average removal of total bacteria measured as 16S rRNA gene (**Figure 2.1, Table S2.3**), meaning that the average relative abundance of ARGs after treatment did not increase (**Figure 2.2 and Table S2.4**). We observed a 1.76 ± 0.40 log reduction for 16S, which implies a decrease of 98.2% on average. The highest removal rates were observed for *qnrS*, *ermB* and *tetM* genes (2.65 ± 0.68, 2.65 ± 0.74, 2.53 ± 0.68 average log reduction respectively*).* For $bla_{CTX\text{-}M}$, 16% of the effluent samples had concentrations below the detection limit, and the average removal excluding those samples was 2.44 ± 0.56 logs. The *sul2* removal was 2.00 ± 0.48 log copies, and closer to the 16S rRNA gene removal was *sul1* with 1.82 ± 0.53 log copies reduction. Similar values were obtained for the MGE *intI1* (1.80 ± 0.49). These results are comparable or better to those obtained in WWTPs including advance treatments or disinfection processes (Di Cesare et al., 2016; Laht et al., 2014; Wen et al., 2016) as described in **Table S2.3**. On the other hand, lower average reduction than the 16S rRNA gene was observed for the *korB* gene, with 0.89 ± 0.60 log removal (87.2%), meaning that the relative abundance of this gene was significantly increased ($p<0.001$) after the treatment (**Figure 2.2, Table S2.4**). Since broad host range plasmids as IncP-1 are known to disseminate into a great variety of environmental bacteria families, their removal might be countered by HGT events (Bellanger et al., 2014; Klümper et al., 2015). In addition, IncP-1 plasmids often include genes that encode for metal

resistance and the degradation of xenobiotic compounds, thus, conferring metabolic advantages to bacteria in activated sludge and likely enhancing their dissemination (Dröge et al., 2000). The presence of IncP-1 plasmids in WWTPs has been previously investigated by culturing and molecular-based techniques (Bahl et al., 2009; Moura et al., 2010), but to the best of our knowledge, this is the first time that their occurrence has been quantified, revealing an increase in their relative abundance after wastewater treatment.

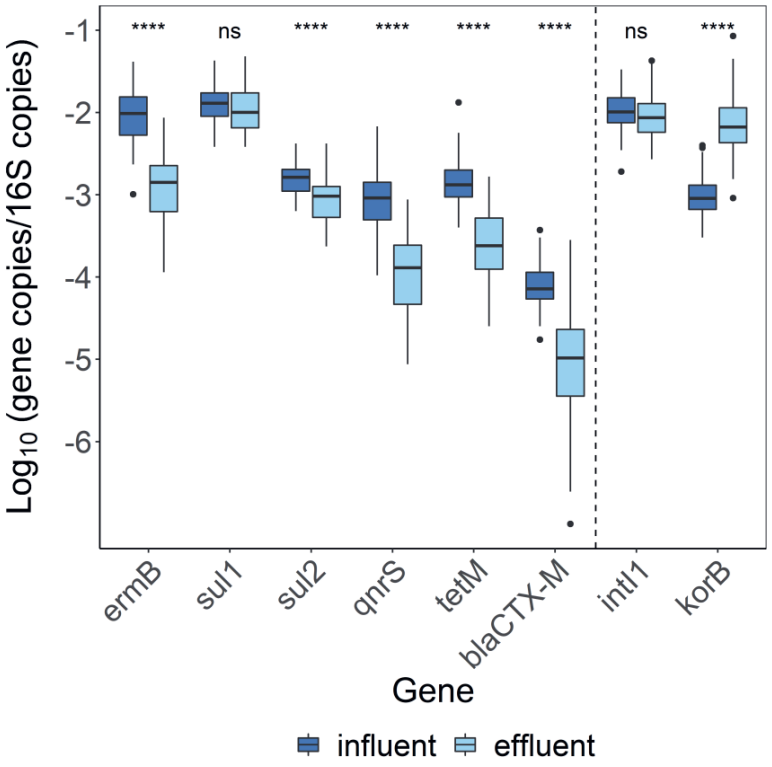


Figure 2.2. Relative abundance of ARGs and MGEs relative to 16S rRNA gene in the influent and effluent of 62 Dutch WWTPs. Different types of genes (16S, ARGs and MGEs) are separated by a vertical line for better interpretation of the plot. The Results are expressed in $\log_{10}$ copies mL^{-1}. The boxes represent the 2nd and 3rd quartiles. The middle line in black represents the median, and the whiskers represent 1st and 4th quartile. Significant differences observed after treatment on each gene were tested by a paired Wilcoxon test, and values are expressed above each gene (****): Highly significant, ($p<0.0001$); ns: no significant differences observed.

Generally, it can be concluded that Dutch WWTPs do not contribute to enhancing antimicrobial resistance. The absolute concentration of the tested ARGs and of *intI1* genes decreased on average 98.4-99.8% after treatment. Besides, the average relative abundance of ARGs either decreased or remained identical (although, a slight relative increase was found in some plants for *sul1, sul2* and *intI1*, **Table S2.1**). Most of the available studies agree that WWTPs reduce the absolute numbers of both total bacteria and ARGs in wastewater. Yet, the effect of

treatment on the relative ARG abundance differs greatly depending on the studied genes. For instance, Munir et al. (2011) and Laht et al. (2014) reported a decrease or no change in the relative abundance of the tested ARGs. On the other hand, relative enrichment of some of the tested ARGs have been observed elsewhere (Di Cesare et al., 2016; Lee et al., 2017; Makowska et al., 2016; Rodriguez-Mozaz et al., 2015). Moreover, Rafraf et al. (2016) found an increase of up to 0.50-2.40 logs in the absolute concentration of some ARGs after treatment. The decrease of absolute and relative abundance of ARGs found in this study might be the result of the combination of the low human use of antibiotics in the Netherlands (possibly limiting selective pressures of these substances in sewage) together with continuous surveillance and upgrading of the Dutch wastewater facilities.

Despite the average of 2.30 ±0.30 log removal of resistance genes, Dutch WWTPs still release approximately 10^6 ARG copies per liter of effluent. The impact of the discharge of ARG-containing effluent on the receiving waterbodies was not evaluated in this study, but it has been addressed in previous works (LaPara et al., 2015; Marti et al., 2013; Rodriguez-Mozaz et al., 2015; Sabri et al., 2018); in most of the cases, the discharge of WWTPs effluents increases the ARGs content in the receiving aquatic ecosystems. This illustrates that human exposure to ARG emitted from WWTP is possible, e.g., through recreation in surface waters. The exact public health burden of the presence of specific resistance genes in surface water is difficult to quantify. However, recreational exposure has been linked with higher ESBL carriage in surfers (Leonard et al., 2018).

2.3.3. Removal of ARGs as compared to MGEs, 16S rRNA gene, and E. coli

The removal of all ARGs and MGEs was positively correlated with the removal of the 16S rRNA gene (r = 0.68-0.87), **Figure 2.3**. Moreover, a strong and significant correlation was observed between the removal of *ermB* and *tetM* (r = 0.96, p<0.001). This could be explained by their typical co-location on diverse transposon families (Brenciani et al., 2007) that are usually present in Gram-positive bacteria, mainly in the order Lactobacillales (Park et al., 2010). These bacteria are common fecal microorganisms present in the wastewater influent, and in general, they are partially removed during WWTP treatment (Cai et al., 2014). The removal of another type of fecal bacteria, *Escherichia coli,* was also significantly correlated with the decrease of the beta-lactam gene $bla_{CTX\text{-}M}$ (r= 0.79, p<0.01) in accordance with the co-location of these genes in Enterobacteriaceae (Bradford, 2001). S*ul1* and *intI1* proved to be correlated in their persistence (r = 0.92, p<0.001), in line with the co-location of *sul1* on (clinical) class 1 integrons (*intI1*). Their resilience to treatment may be due to their association with diverse broad

host range plasmids that are horizontally transferred among diverse bacteria (Gillings et al., 2015).

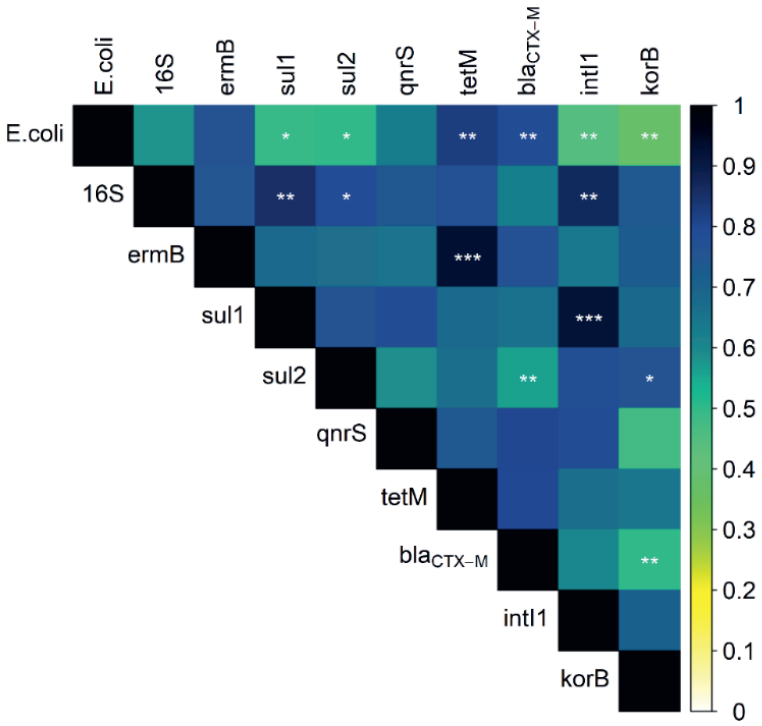


Figure 2.3. Correlation matrix (Pearson's correlation) of the absolute removal efficiency of the diverse ARGs, MGEs, and the proxies 16S rRNA gene and *E. coli*. Significant levels of correlation are indicated as follow p<0.05 (*), p<0.01 (**) and p<0.001 (***). Values from E. coli removal were obtained from Schmitt, 2017 based on the same samples used in this study.

2.3.4. Influence of the design and process parameters of WWTPs in the efficiency of ARGs and MGEs removal

The impact of specific treatment processes on gene removal (as included in **Table 2.2**) was studied by statistical analysis (linear mixed models). These analyses were run both with and without the *korB* gene, as this was the only gene for which the relative abundance increased during wastewater treatment, suggesting a different effect of the treatment dynamics than for the rest of the gene panel. Our results show that an increase of the Hydraulic Load Factor caused by rainfall events was the dominant variable explaining differences in reduction of ARGs and MGEs between the plants (significant effect in the univariate and most multivariate models, both with and without *korB;* **Figure 2.4, Table 2.4, Table 2.5 and Table S2.6)**

On average, the efficiency was reduced by 0.38 logs per time the ADF was exceeded. Therefore, rainfall reduced the incoming loads of ARGs in influent as well as the efficiency of their removal during treatment, yet only the latter was found to be statistically significant.

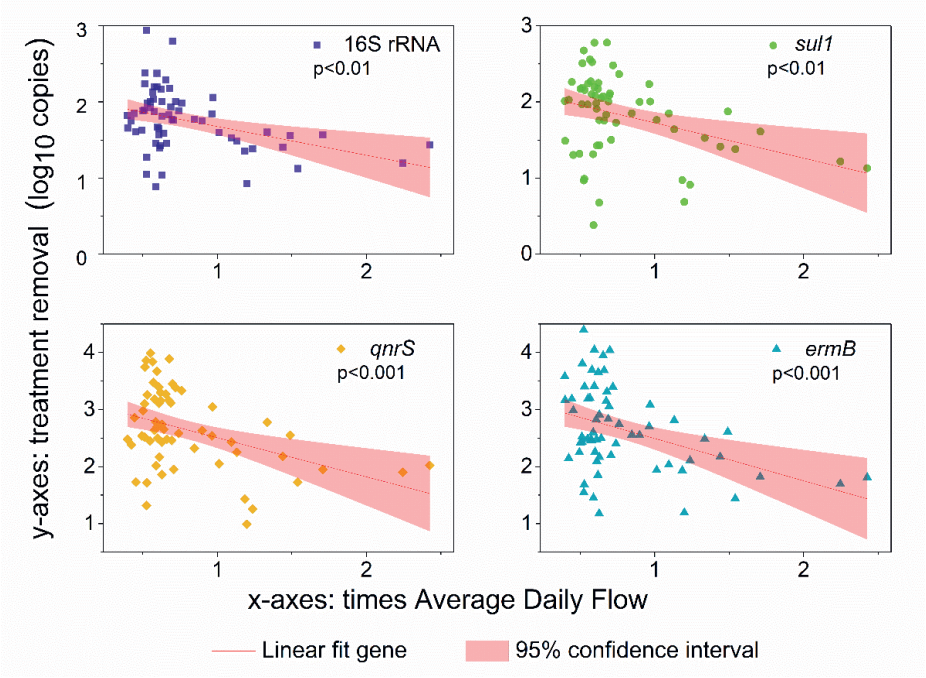


Figure 2.4. Effect of increased Hydraulic Load Factor on the removal efficiency of ARGs. A linear fit of the removal of resistance genes (16S rRNA gene, *ermB*, *sul1*, and *qnrS*, on $\log_{10}$ scale) during WWTP treatment versus Hydraulic Load Factor measured in times the Average Daily Flow (ADF).

WWTPs are usually optimally operated in the so-called Dry Weather Flow, (here represented by the ADF). While wastewater volumes up to a maximum flow of 3 to 6 times the DWF conditions can be processed (EPA, 1995), a higher hydraulic load can disturb the treatment processes. These disturbances are mainly related to the reduction of the WWTP's optimal HRT and differences in the influent composition (Metcalf and Eddy, 2003). In the secondary treatment, shorter HRTs and different influent compositions (physical and biochemical) can affect both biomass growth and the dynamics of biological processes, resulting in poorer treatment performance (Capodaglio, 2007). No significant effects of HRTs on removal rates were found in the univariate analysis, and only a slight effect of HRT was found in multivariate analyses (HRT was included in a few of the best adequate models, but not significant in itself). The increase in removal with 1 h HRT was limited in all these models to 0.01

log gene copies. Yet, shorter HRT occurring during higher hydraulic loads on the sampling day likely contributed to poorer treatment efficiency.

When the increase in the hydraulic load is intense or lasts for a long period, it can cause washing out of activated sludge, leading to an increase in total suspended solids (TSS), including bacteria and ARGs, in the effluent (Capodaglio, 2007; Rouleau et al., 1997). TSS in effluent could also be increased by increasing TSS in the influents caused by rainfall events (Mines et al., 2007). In this study, effluent's TSS were only marginally influencing ARGs removal in multivariate analysis and not significant in univariate analysis (**Table 2.4**). This was likely due to the use of annual average TSS values, therefore not reflecting the sampling day. However, TSS values from the sampling day were not significantly correlated with ARG concentrations in effluents in a recent study (Ben et al., 2017). Those samplings were performed under dry weather conditions, though. Therefore, the association between TSS and WWTP's removal efficiency during rainy weather events still remains unclear, and it is advisable to investigate the influence of this parameter in future studies.

Table 2.4. Effect of operational parameters on the reduction of ARGs and MGEs (univariate models excluding *korB*). Models give the effect of single operational parameters, adjusted for the Hydraulic Load Factor and its interaction with the gene identity. Acronyms: n: number of plants with available information for that parameter; beta: model estimate; SE: standard error of the estimate; z: z statistics; p: p-value; NP: no primary clarification; P: primary clarification without reject water; PR: Primary clarification with recirculation of reject water; B: biological phosphorus (P) removal; C: Chemical P removal; BC: Biochemical P removal. HRT: Hydraulic Retention Time; SRT: Sludge retention time; ACT: Anaerobic Contact Time. The model for the Hydraulic Load Factor includes the Hydraulic Load Factor, the gene, and their interaction only. In all models, *ermB, tetM,* and *qnrS* are significantly better removed than 16S rRNA. In some models, *sul2* is also significantly better removed than 16S rRNA. An increase in the Hydraulic Load Factor leads to significantly reduced removal for *ermB, tetM,* and *qnrS* as compared to 16S rRNA in all univariate models.

			n	beta	SE	Z	p
Factor	Hydraulic Load Factor		62	-0.38	0.17	-2.23	0.03
	Size P.E. (150 g TOD)		62	-1.55E-07	3.64E-07	-0.43	0.67
	Primary settling		62				0.42
		NP	44				
		P	10	-0.15	0.17	-0.92	0.36
		PR	8	-0.19	0.18	-1.05	0.30
	P removal	none	23				0.74
		B	18	0.10	0.15	0.65	0.52
		C	7	0.23	0.21	1.11	0.27
		BC	14	0.03	0.16	0.19	0.85
	HRT [hours]		45	0.01	0.01	1.33	0.19
	SRT [hours]		51	0.01	0.01	1.00	0.32
	ACT [hours]		11	0.14	0.15	0.42	0.36
	Effluent TSS [mg/L]		62	-0.02	0.01	-1.52	0.13

Average SRT only slightly affected gene removal in multivariate analysis and was not significant in the univariate analysis. The SRT is optimized in each plant to achieve the best conditions for nutrient removal (Smith et al., 2014). Although higher SRTs have been shown to improve the removal of pharmaceuticals (De Sotto et al., 2016), its effect on ARB and ARGs removal is still controversial and restricted to bench-scale studies (De Sotto et al., 2016; Neyestani et al., 2017). Higher SRT might favour the grazing of bacteria by protozoa, but on the other hand, it might also promote HGT events (Tsutsui et al., 2010). Lastly, we also evaluated the effect of primary sedimentation processes. These are meant to reduce debris, TSS, and BOD by mechanical and/or settling procedures (Puig et al., 2010). Bacteria associated with such particles might be removed during primary settling. However, such an effect may be masked by primary settlers receiving recirculated reject water from thickeners that contains high amounts of ARGs (Gao et al., 2012). In our study, the presence of primary sedimentation with and without recirculation of digested sludge seemed to result in slightly decreased ARG removal. This appeared from the inclusion of this parameter in the two best models, retrieved on a subset of the data with complete observations, albeit these parameters not being significant in themselves (**Table 2.4 and Table S2.5**). However, when this statistical model was repeated on the full dataset, the effect of primary sedimentation with and without recirculation of digested sludge was less pronounced (i.e., the estimates were numerically smaller and the p-values higher) (**Table 2.5 and Table S2.6**). Last, the effects of the remaining design and operational WWTPs parameters investigated (**Table 2.2**) were not statistically significant, namely the size of the plant and the type of P removal (chemical or biological) (**Table 2.4**).

Thus, the increased hydraulic load caused by rainfall remains the single clear parameter of the dataset determining the ARG removal. The simplest model describing resistance gene reduction, therefore, includes the hydraulic load only (**Table S2.7 in the supplementary information**). According to this model, *ermB, sul2, qnrS,* and *tetM* are removed significantly more efficiently than 16S rRNA, while *korB* increases in relative abundance. The effect of the hydraulic load on the removal efficiency differs per gene: *qnrS, tetM,* and *ermB* are significantly worse removed at higher hydraulic load (from -0.69 to -0.83 logs per time ADF was doubled) as compared to 16SrRNA (-0.38 logs) **(Table S2.7)**

Table 2.5. Effect of operational parameters on the reduction of ARGs and MGEs (excluding *korB*) – results of multivariate modeling. The estimates of the effects of explanatory variables on gene removal (on $\log_{10}$ scale) during WWTP treatment (p values are shown in italic between brackets; significant estimates are shown in bold) are shown for the 11 best models that were of nearly identical quality, as determined by AICc. For each model, both the results based on the subset of 37 plants for which all parameters were known and the results for the larger subset of plants for which these specific model parameters were known are given. The number of plants included in the model is shown under "n". Gene identity was also included in all models, as was the interaction between the Hydraulic Load Factor (HLF) and

the gene identity. Genes for which significant interactions with the HLF were found are listed (for *ermB, qnrS,* and *tetM*, the decrease in log removal with increasing HLF was more pronounced than for 16S rRNA). Acronyms: n: umber of plants in the model; p: p-value NP: no primary clarification; P: primary clarification without recirculation of reject water; PR: Primary clarification with recirculation of reject water; N: not included in the model.

Inter-cept	Hydraulic Load Factor	E.TSS	HRT	Primary clarifier	SRT	Interaction between Hydraulic Load Factor : gene	AICc	n
2.23	-0.59 *(p<0.001)*	N	N	N	N	N	232.4	37
2.20	-0.56 *(p<0.001)*	N	N	N	N	N		62
2.34	-0.63 *(p<0.001)*	N	N	P: -0.33 *(p=0.12)* PR: -0.56 *(p=0.23)*	N	N	232.8	37
2.24	-0.56 *(p<0.001)*	N	N	P: -0.15 *(p=0.36)* PR: -0.19 *(p=0.30)*	N	N		62
2.15	-0.58 *(p=0.002)*	N	0.01 *(p=0.21)*	N	N	N	232.8	37
2.13	-0.56 *(p=0.002)*	N	0.01 *(p=0.19)*	N	N	N		45
2.07	-0.38 *(p=0.066)*	N	N	N	N	*ermB, qnrS, tetM*	233	37
2.05	-0.38 *(p=0.028)*	N	N	N	N	*ermB, qnrS, tetM*		62
2.34	-0.59 *(p=0.002)*	-0.01 *(p=0.31)*	N	N	N	N	233.5	37
2.34	-0.58 *(p<0.001)*	-0.02 *(p=0.13)*	N	N	N	N		62
1.99	-0.37 *(p=0.071)*	N	0.01 *(p=0.21)*	N	N	*ermB, qnrS, tetM*	233.6	37
1.97	-0.36 *(p=0.080)*	N	0.01 *(p=0.19)*	N	N	*ermB, qnrS, tetM*		45
2.17	-0.42 *(p=0.039)*	N	N	P: -0.33 *(p=0.12)* PR: -0.56 *(p=0.23)*	N	*ermB, qnrS, tetM*	233.6	37
2.1	-0.38 *(p=0.029)*	N	N	P: -0.30 *(p=0.12)* PR: -0.57 *(p=0.23)*	N	*ermB, qnrS, tetM*		62
2.18	-0.39 (p=0.063)	-0.01 *(p=0.31)*	N	N	N	*ermB, qnrS, tetM*	234.2	37
2.19	-0.40 (p=0.020)	-0.02 *(p=0.13)*	N	N	N	*ermB, qnrS, tetM*		62
2.25	-0.58 (p=0.002)	-0.01 *(p=0.38)*	0.01 *(p=0.25)*	N	N	N	234.2	37

Inter-cept	Hydraulic Load Factor	E.TSS	HRT	Primary clarifier	SRT	Interaction between Hydraulic Load Factor : gene	AICc	n
2.25	-0.56 (p=0.002)	-0.01 *(p=0.26)*	0.01 *(p=0.24)*	N	N	N	234.3	45
2.27	-0.62 *(p=0.001)*	N	0.01 *(p=0.39)*	P: -0.29 *(p=0.18)* PR: -0.53 *(p=0.27)*	N	N		37
2.18	-0.58 *(p=0.002)*	N	0.01 *(p=0.28)*	P: -0.09 *(p=0.64)* PR: -0.26 *(p=0.46)*	N	N		45
2.10	-0.57 *(p=0.004)*	N	N	N	0.01 *(p=0.60)*	N	234.3	37
2.05	-0.55 *(p<0.001)*	N	N	N	0.01 *(p=0.32)*	N		51

2.3.5. The challenge of comparing and anticipating treatment efficiencies

Albeit an increasing number of studies addressing antibiotic resistance in WWTPs in the last decade, no conventional treatment or operational strategy has been identified that can improve ARG removal. Unlike in laboratory approaches, full-scale studies involve dozens of variables at once. Effects caused by parameters that are targeted in a specific study can be masked by others (environmental, design). As the majority of the available studies include relatively few locations (from 1 to 5 WWTPs), meta-analyses might be used to aggregate data from single studies and more sensitively identify explanatory factors for ARG removal. However, some studies do neither gather nor include crucial metadata about plant design and operational parameters along with the sampling campaign. Preferably, the collected parameters should be specific for the sampling dates rather than representing average values. In any case, cooperation with water authorities in both the sampling design and the evaluation of the results might ease the access to operational process information. Furthermore, the comparison of results between studies is not always possible since the ARGs assessed often differ. This might be helped by a consensus panel of ARGs, such as suggested by Berendonk et al. (2015), which was used in this work. Additionally, not all studies report both reductions in absolute concentrations and reduction of the relative abundance of ARGs normalized to the 16S rRNA gene. The absolute ARGs concentrations in influent or discharged effluents provide valuable information for risk assessments and to estimate the plant performance. On the other hand, relative abundance is relevant to point to possible selective processes within the plant.

Integrating each of the aforementioned points into future studies would help to build a comprehensive data frame that might result in a better understanding of the efficiency of ARG removal in wastewater treatment.

2.4. Conclusions

From an analysis of the influent concentrations and the removal of ARGs in a large number of WWTPs in the Netherlands, we conclude that:

- From the studied ARGs, *sul1* (sulphonamide resistance) and *ermB* (macrolide resistance) are the most predominant resistance genes in the influent of WWTPs.
- The presence of known sources of antibiotic reistance in catchment areas, namely healthcare institutions (hospitals, nursing homes, or polyclinics), only marginally influences concentrations of ARGs and MGEs in influent of municipal WWTPs.
- Conventional WWTP treatment significantly decreased the absolute numbers of total bacteria and the investigated ARGs by 1.76-2.65 logs. Moreover, the treatment did not increase the relative abundance of the tested ARGs. However, Dutch WWTPs still discharge on average 10^6 copies of ARGs per liter of effluent to the receiving water bodies.
- This study quantifies for the first time IncP-1 plasmids (measured as *korB* gene) in wastewater samples finding that its relative abundance significantly increased after WWTP treatment.
- Rainfall causing an increase in the usual WWTP hydraulic load marginally reduced the amount of incoming ARGs but significantly reduced the WWTP's removal efficiency of the ARGs and *intI1*.
- WWTP's design parameters as size, presence of primary clarification, type of P removal and operational parameters as HRT, SRT, anaerobic contact time and effluent TSS were not found to affect the removal of the studied ARGs and MGEs significantly. However, the use of average annual data instead of actual data on the sampling day for some of these parameters probably masked their possible effect.

2.5. Acknowledgements

We would like to thank all the Dutch waterboards and WWTP operators for their collaboration in the sampling collection. We also warmly acknowledge Dr. Sven Jechalke and Dr. Kornelia Smalla from the Julius Kühn-Institut in Germany for providing the controls for the

IncP-1 (*korB)* qPCR assay. Finally, we would like to thank Adrián González Fraile for his help during the setup of the *korB* assay.

This work was partly funded by the Ministry VWS to RIVM. Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslan and the Northern Netherlands Provinces. Besides, this research has received funding from the European Union's Horizon2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 665874. The authors would also like to thank the members of the research theme Source Separated Sanitation for the shared knowledge and financial support.

2.6. Supplementary information

Figure S2.1: WWTPs sampled in this study. The map (grey) represents The Netherlands. Each pin (blue) corresponds to one sampling location (WWTP location) from a total of 62 sampling points. Further information about the sampled WWTPs is detailed in **Table S2.1.**

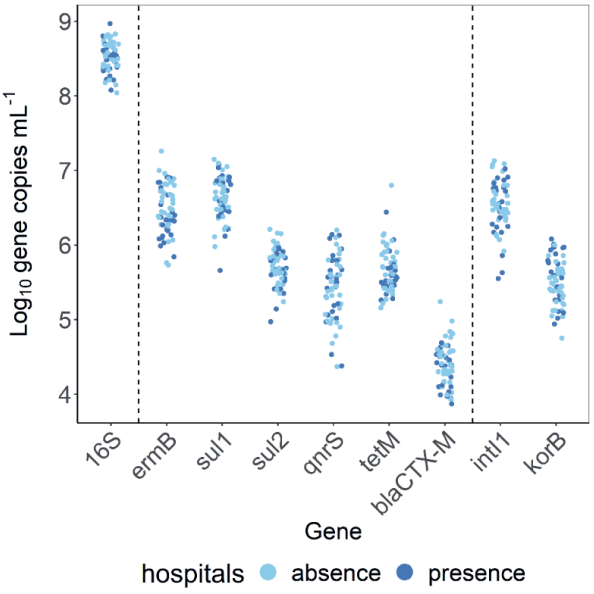


Figure S2.2. Absolute concentrations of resistance genes and mobile genetic element genes in the influents of 62 WWTPs. Results are expressed in $\log_{10}$ copies per mL of influent. No significant differences ($p>0.05$) were observed between WWTPs that treat hospital wastewater (dark blue) and WWTP not treating hospital wastewater (light blue).

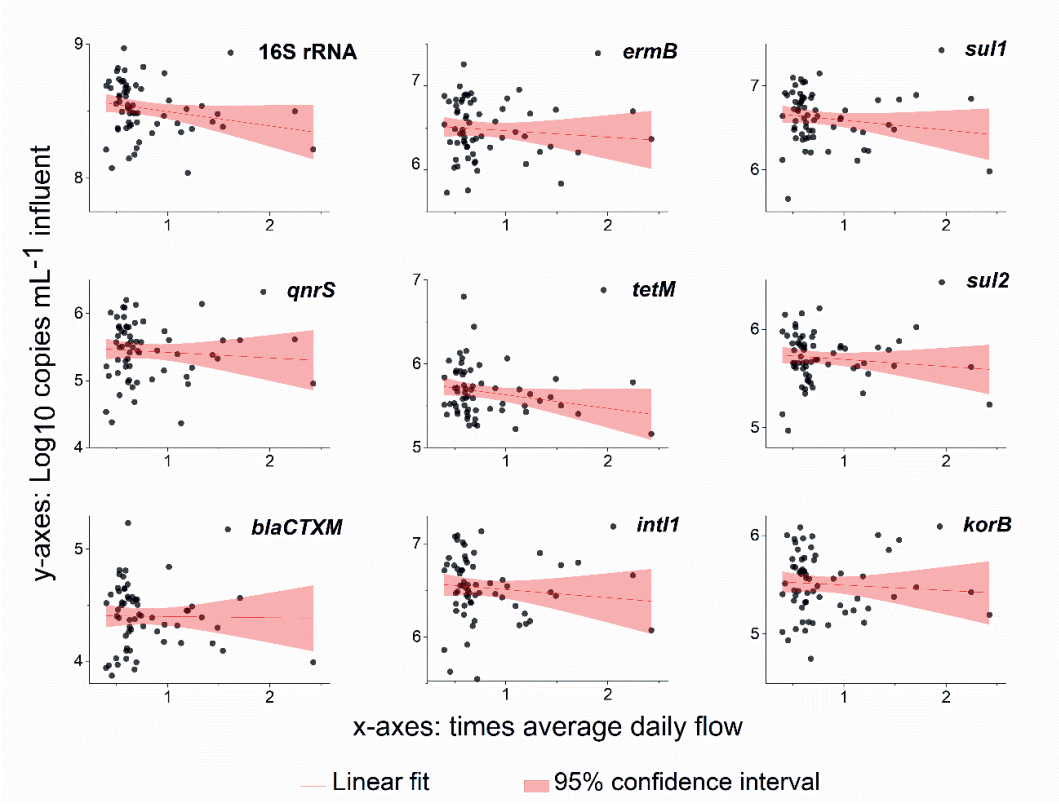


Figure S2.3. Effect of an increased hydraulic load factor on influent concentrations of 16S, ARGs, and MGEs. Gene concentrations are expressed in log_{10} copies per mL of influent and the increased hydraulic load factor in times the average daily flow. The slopes were not significantly different from zero ($p>0.05$).

Table S2.1. Design capacity in PE, process configuration, and log_{10} removal of ARGs and MGEs in 62 Dutch wastewater treatment plants (WWTPs). WWTP characteristics were retrieved from the respective water boards. The gene removal is given as the decadic logarithm of the ratio between the influent and effluent concentration). Acronyms: NA: data not available; TOD: Total oxygen demand; Prim Sed: Primary Sedimentation; C; Carbon; N: Nitrogen; BioP: Bio Phosphorus removal; Chem P: Chemical Phosphorus removal.

WWTP number	Size in PE (150g TOD)	Primary treatment			Secondary treatment							Gene removal (log_{10})								
					Steps present			Nutrient removal												
		Screens	Others	Prim Sed.	Anae	Anox	Oxic	C	N	BioP	ChemP	*16S*	*ermB*	*sul 1*	*sul2*	*tetM*	*qnrS*	*bla$_{ctxM}$*	*intI1*	*korB*
1	564000	x	GC	x			x	x	x		x	2.00	3.40	2.48	2.60	3.22	3.39	*NA*	1.59	0.91
2	56247	x	GC		x	x	x	x	x	x		1.36	1.93	0.97	1.58	1.79	1.43	1.74	1.19	0.78
3	13421	x	ST		x	x	x	x	x	x		1.20	1.69	1.22	1.12	1.42	1.90	1.96	1.24	0.35
4	121408	x	ST	x	x	x	x	x	x	x	x	1.38	2.10	0.91	1.63	2.02	1.26	1.06	1.09	0.46
5	44427	x			x	x	x	x	x	x		1.99	3.40	2.16	2.04	3.23	3.26	3.53	2.25	1.05
6	66187	x			x	x	x	x	x	x		1.82	3.58	2.01	1.67	3.21	2.48	2.95	1.40	0.91
7	62560	x			x	x	x	x	x	x		2.29	3.95	2.08	2.89	3.88	3.27	3.21	2.45	1.97
8	54.400	x		x				x	x		x	1.66	3.17	1.49	2.38	3.24	2.48	3.19	1.58	0.87
9	114240	x				x	x	x	x		x	2.17	3.65	2.25	2.61	3.15	3.16	3.41	1.90	1.77
10	137573	x		x	x	x	x	x	x	x		1.77	3.05	2.08	2.28	2.55	2.46	2.98	1.95	1.47
11	31733	x			x	x	x	x	x	x		2.38	3.41	2.78	2.6	3.23	3.67	*NA*	2.80	1.41
12	168820	x	x		x	x	x	x	x	x	x	1.77	2.56	1.85	2.08	2.53	2.32	2.61	1.94	0.91
13	40710	x			x	x	x	x	x	x		1.84	3.15	1.83	2.16	3.16	3.16	2.55	1.83	0.67
14	165010	x	x	x	x	x	x	x	x	x		1.61	2.98	1.30	1.53	2.60	1.73	2.29	1.20	0.85
15	680000	x		x	x	x	x		x	x	x	1.76	2.20	1.50	1.87	1.87	1.95	2.05	1.66	1.21
16	340000	x		x	x	x	x		x	x	x	2.80	4.04	2.11	3.12	3.69	3.45	*NA*	2.20	2.60
17	72533	x	GC		x	x	x	x	x	x		0.93	1.19	0.68	1.16	1.15	0.99	1.08	0.80	0.15
18	165217	x	GC		x	x	x	x	x	x	x	1.81	2.17	1.43	2.07	2.07	2.74	2.47	1.48	1.37
19	57301	x	ST			x	x	x	x		x	2.20	4.05	2.20	2.38	3.52	2.70	3.06	2.32	1.72

WWTP number	Size in PE (150g TOD)	Primary treatment			Secondary treatment							Gene removal (log_{10})								
					Steps present			Nutrient removal												
		Screens	Others	Prim Sed.	Anae	Anox	Oxic	C	N	BioP	ChemP	*16S*	*ermB*	*sul 1*	*sul2*	*tetM*	*qnrS*	*bla$_{ctxM}$*	*intI1*	*korB*
20	7444	x			x	x	x	x	x	x		2.94	4.39	2.68	2.69	4.07	3.86	*NA*	3.04	2.12
21	77973	x	ST	x		x	x	x	x		x	2.20	3.70	2.53	2.21	3.37	3.18	*NA*	2.50	1.30
22	24734	X	GC		x	x	x	x	x	x		1.98	2.74	2.37	2.26	2.65	3.33	2.53	2.45	0.88
23	4819	x				x	x	x	x		x	1.04	1.18	0.68	1.13	1.37	1.86	1.69	0.90	-0.22
24	78900	x			*NA*	*NA*	*NA*	x	x	x		1.76	2.15	2.03	2.46	2.34	2.38	2.36	2.13	0.37
25	73500	x			*NA*	*NA*	*NA*	x	x	x		1.56	2.48	1.91	2.18	2.36	3.11	3.37	1.87	0.06
26	90000	x		x	*NA*	*NA*	*NA*	x	x	x		1.05	1.55	0.97	1.36	1.16	1.32	1.59	1.00	0.24
27	86400	x		x	*NA*	*NA*	*NA*	x	x	x		1.63	2.25	1.32	1.77	2.36	2.54	2.13	1.36	-0.09
28	14000	x			*NA*	*NA*	*NA*	x	x			1.89	2.52	1.97	1.97	2.44	2.52	2.35	1.88	0.73
29	258000	x		x	x	x	x	x	x	x	x	1.12	1.44	1.38	1.23	1.49	1.73	1.45	1.34	0.23
30	1060482	x		x	x	x	x	x	x	x		1.60	2.48	1.53	1.43	2.19	2.78	2.66	1.72	0.42
31	344993	x		x	x	x	x	x	x	x		1.56	1.82	1.61	1.64	1.45	1.95	1.95	1.52	0.54
32	10880					x	x	x	x		x	1.41	2.17	1.41	1.83	1.74	2.18	1.85	1.61	1.21
33	73405	x	x	x		x	x	x	x		x	1.67	2.26	2.08	2.06	2.15	2.50	2.33	2.01	0.95
34	57347	x	ST		x	x	x	x	x	x		1.94	2.83	2.01	2.42	2.83	3.12	2.60	2.29	1.45
35	NA	x			x	x	x	x	x	x		1.85	3.19	2.26	2.44	2.96	2.86	2.70	2.29	1.36
36	340146	x	x			x	x	x	x		x	0.89	1.45	0.38	0.61	1.88	2.02	2.51	0.39	-0.59
37	7661	x				x	x	x	x		x	1.45	2.60	1.31	2.01	2.36	2.79	2.49	1.31	0.65
38	74347	x	ST	x		x	x	x	x		x	1.28	1.69	0.99	1.55	1.49	1.72	1.79	1.10	-0.27
39	14053	x	ST		x	x	x	x	x	x		1.39	2.09	1.43	1.77	2.01	2.17	2.52	1.51	0.58
40	83413	x			x	x	x	x	x	x		2.03	2.46	2.27	2.29	2.41	3.48	2.80	2.35	1.05
41	398933	x	ST	x	x	x	x	x	x	x	x	1.45	3.69	1.76	1.82	3.19	2.48	2.72	1.69	0.84

WWTP number	Size in PE (150g TOD)	Primary treatment			Secondary treatment							Gene removal (log_{10})								
					Steps present			Nutrient removal												
		Screens	Others	Prim Sed.	Anae	Anox	Oxic	C	N	BioP	ChemP	*16S*	*ermB*	*sul 1*	*sul2*	*tetM*	*qnrS*	*bla_{ctxM}*	*intI1*	*korB*
42	98373	x			x	x	x	x	x	x	x	2.18	3.31	2.78	2.50	3.38	3.89	*NA*	2.59	1.57
43	8432	x					x	x	x			1.42	1.85	2.17	1.42	2.04	3.30	*NA*	2.15	-0.04
44	25610	x			x	x	x	x	x	x	x	2.38	3.81	2.51	2.54	3.60	3.75	*NA*	1.68	2.13
45	9800	x			*NA*	*NA*	*NA*	x	x			2.24	2.43	2.19	2.05	2.93	3.10	2.71	2.11	0.82
46	11787	x					x	x	x		x	1.88	2.40	1.73	1.89	2.46	2.58	2.24	1.84	0.42
47	13600	x					x	x	x			1.87	2.46	2.10	2.35	2.69	2.64	2.05	1.92	1.01
48	41707	x					x	x	x			1.75	2.55	2.00	2.52	2.54	2.63	2.37	2.03	1.22
49	7797	x			x		x	x	x			2.00	2.49	1.97	2.42	2.43	2.45	2.55	2.08	1.19
50	30192	x		x			x	x	x			1.93	3.19	2.24	1.48	3.2	3.99	3.27	2.28	0.88
51	74709	x	x			x	x	x	x		x	1.65	2.83	1.98	2.08	2.87	3.4	3.44	1.90	0.96
52	289136	x	x			x	x	x	x		x	1.59	2.49	2.05	1.92	2.55	2.65	2.48	1.60	0.7
53	147787	x	x	x		x	x	x	x		x	2.01	2.91	1.76	2.11	2.46	2.43	2.30	1.89	1.11
54	57845	x	x	x			x	x	x		x	2.06	3.09	2.01	2.74	2.93	3.05	3.03	1.99	1.47
55	10842	x				x	x	x	x		x	1.88	2.42	2.17	1.68	2.57	2.98	2.38	2.42	0.87
56	101248	x	x		x	x	x	x	x	x		2.13	3.20	2.56	2.56	3.3	3.84	*NA*	2.16	1.22
57	7797						x	x	x			1.44	1.81	1.13	1.42	1.68	2.02	1.75	1.38	0.64
58	9611						x	x	x			1.60	1.94	1.76	1.81	1.91	2.05	2.44	1.58	0.62
59	13147						x	x	x			1.49	2.81	1.64	1.7	2.82	2.25	2.75	1.59	0.58
60	10427	x					x	x	x			1.53	2.03	1.85	1.87	1.98	2.43	2.10	1.70	0.83
61	7072	x					x	x	x		x	1.56	2.61	1.87	2.22	2.46	2.55	3.00	1.92	1.23
62	13600	x			x		x	x	x			1.84	2.70	2.24	2.09	2.00	2.54	2.31	1.92	0.58

qPCR: oligonucleotides, probes, and reaction and conditions

All ARGs and *intI1* qPCR reactions were conducted in 20 µL, including IQ™ SYBR green supermix BioRad 1x, and BSA 0.8 mg/mL (m/v) (Sigma Aldrich, NL). Forward and reverse primers concentrations, and oligonucleotide probes (when applicable) are summarized in **Table S1.** A total of 2 µL of DNA template was added to each reaction, and the reaction volume was completed to 20 µL with DNase/RNase free Gibcowater (Life technologies, Lithuania). All reactions (except for the *korB* gene) were performed in a CFX96 Touch ™ thermocycler from BioRad (NL) according to the following PCR cycles: 95ºC for 5 min followed by 40 cycles at 95ºC for 15 s and 60ºC for 30 s. The annealing temperature was the same for all the different reactions except for the s*ul2* and *sul1* genes. In those cases, the annealing temperatures were 61ºC and 65ºC, respectively. *korB* gene's reaction was conducted according to Jechalke et al. (2013).

In order to check the specificity of the reaction, a melting curve was performed from 65 to 95ºC at a temperature gradient of +0.5ºC (5 s)$^{-1}$. Synthetic DNA fragments (IDT, US) containing each of the target genes were used as a positive control to create the standard curves. Serial dilutions of gene fragments were performed in sheared salmon sperm DNA 5 µg mL^{-1} (m/v) (Thermofisher, LT) diluted in Tris-EDTA (TE) buffer at pH 8.0 (Sigma Aldrich, Switzerland). Every sample was analyzed in technical duplicates. Standard curves were included in each PCR plate with at least 5 serial dilutions points and in technical duplicate. An average standard curve based on a standard curve from every run was created for every gene set. Gene concentration values were then calculated from the aforementioned curve.

Table S2 2. Oligonucleotides and probes used for ARGs and MGEs detection by qPCR reactions. In primes/probes with degenerate code, Y stands for pyrimidine bases (C or T), R stands for purines (A or G), S for strong bases (C or G) and V for A,C,G (IUPAC nomenclature).

Target gene	Reference	Probe name	Oligonucleotide sequence 5'-3'	Concen in reaction (nmol L^{-1})
16S rRNA	(Lane, 1991; Muyzer et al., 1993)	338F	ACTCCTACGGGAGGCAGCAG	300
		518R	ATTACCGCGGCTGCTGG	
qnrS	(Marti et al., 2013)	qnrSrtF11	GACGTGCTAACTTGCGTGAT	400
		qnrSrtR11	TGGCATTGTTGGAAACTTG	
tet(M)	(Peak et al., 2007)	tet(M)F	GGTTTCTCTTGGATACTTAAATCAATCR	500
		tet(M)R	CCAACCATAYAATCCTTGTTCRC	
sul1	(Pei et al., 2006)	Sul1-F	CGCACCGGAAACATCGCTGCAC	300
		Sul1-R	TGAAGTTCCGCCGCAAGGCTCG	
sul2	(Pei et al., 2006)	Sul2-F	TCCGGTGGAGGCCGGTATCTGG	400
		Sul2-R	CGGGAATGCCATCTGCCTTGAG	
erm(B)	(Knapp et al., 2010)	ErmB-F	AAAACTTACCCGCCATACCA	400
		ErmB-R	TTTGGCGTGTTTCATTGCTT	
bla_{ctxM}	(Marti et al., 2013)	q_CTXM-F	CTATGGCACCACCAACGATA	400
		q_CTXM-R	ACGGCTTTCTGCCTTAGGTT	
intI1	(Barraud et al., 2010)	IntI-F	GATCGGTCGAATGCGTGT	400
		IntI-R	GCCTTGATGTTACCCGAGAG	
korB (IncP-1)	(Jechalke et al., 2013)	IncP-F	TCATCGACAACGACTACAACG	300
		IncP-Fz	TCGTGGATAACGACTACAACG	
		IncP-R	TTCTTCTTGCCCTTCGCCAG	
		IncP-Rd	TTCTTG ACTCCCTTCGCCAG	
		IncP-Rge	TTYTTCYTGCCCTTGGCCAG	
		Probe-P	TCAGYTCRTTGCGYTGCAGGTTCTCVAT	
		Probe-Pgz	TSAGCTCGTTGCGTTGCAGGTTYUCAAT	

Table S2.3. Treatment characteristics and ARG presence and removal observed in this study (n=62) and other recent studies. Values are expressed in log_{10}. Prevalence values of influent (I) and effluent (E) were obtained from the available data included in the corresponding publications. When not available, data were obtained by plot digitalization with Origin Pro 2014 software (a), or by hand (b) from plots in the corresponding publications. An estimation of the removal values was performed by subtracting influent and effluent values. Size in PE is in 54g BOD. Acronyms are as follow: Advan: Advanced treatment; SD: Standard Deviation; GC: Grit chamber; PC: Primary clarifier; SC: Secondary clarifier; CAS: Conventional activated sludge; CASS: cyclic activated sludge system, P+M+B: Physical + Mechanical + Biofilters; I: influent; E: effluent. NL: Netherlands, TN: Tunisia, IT: Italy, PL: Poland; CN: China; ES: Spain; FI: Finland; EE: Estonia; CH: Switzerland.

Reference	Location	WWTP	Size (in PE)	Treatment					Sample	Average absolute log_{10} gene copies mL^{-1}								Average log Reduction = Log_{10} (gene copies I / gene copies E)							
				Primary	Secondary		Advan.			16S	*ermB*	*sul1*	*sul2*	*tetW*	*tetM*	*qnrS*	*bla* CTX-M	*intI1*	16S	*ermB*	*sul1*	*sul2*	*tetW*	*tetM*	*qnrS*	*bla* CTX-M	*intI1*
This study	NL	62	10^2-10^6	Varied	Varied		None		I	**8.52**	**6.48**	**6.62**	**5.71**		**5.66**	**5.44**	**4.40**	**6.53**	**1.76**	**2.65**	**1.82**	**2.00**		**2.53**	**2.65**	**2.40**	**1.80**
									SD	*0.40*	*0.33*	*0.29*	*0.24*		*0.29*	*0.42*	*0.28*	*0.33*	*0.40*	*0.74*	*0.53*	*0.48*		*0.68*	*0.68*	*0.64*	*0.50*
									E	**6.77**	**3.84**	**4.80**	**3.71**		**3.13**	**2.79**	**1.94**	**4.72**									
									SD	0.40	0.72	0.51	0.48		0.69	0.66	0.61	0.45									
Rafraf et al., 2016	TN	1	$2x10^5$	NA		Aerated lagoons	NA	None	I	8.36	5.24	7.24						7.13	-0.20	-0.61	0.90					0.46
									E	8.56	5.85	6.34				4.38		6.67								
		2	$1x10^4$	NA		CAS	NA	None	I	8.53	6.10	6.24				3.51		6.04	0.34	1.20	-0.55					-0.12
									E	8.19	4.90	6.79						6.16								
		3	$1x10^5$	NA		CAS	NA	None	I	8.77	6.59	7.32				4.10	3.01	6.23	0.57	1.31	0.59			-2.26	-2.41	-0.50
									E	8.20	5.28	6.77				6.36	5.42	6.73								
		4	$2x10^4$	NA		CAS	NA	UV	I	8.35	6.04	6.48				3.52		6.44	0.19	0.93	0.03					0.06
									E	8.16	5.11	6.45						6.38								
		5	$9x10^4$	NA		CAS	NA	None	I	8.64	7.39	6.94				5.34	5.41	6.75	0.29	1.80	0.44			0.29	0.23	0.25
									E	8.35	5.59	6.50				5.05	5.18	6.50								
Di Cesare et al., 2016	IT	1	$5x10^4$	GC	PC	Bio +Chem	SC	Chlorin	I		5.89		5.53			6.09		5.71		3.62		2.79			3.49		2.96
									E		2.28		2.73			2.60		2.74								
		2	$2x10^4$	GC	PC	Biol +Chem	SC	Peracetic acid	I		6.66		5.85			6.78		5.99		3.35		1.78			2.96		2.00
									E		3.32		4.08			3.82		3.99								
		3	$1x10^5$	GC	PC	Bio-Chem	SC	UV	I		5.83		5.25			5.90		5.47		2.56		1.78			2.74		1.58
									E		3.26		3.47			3.16		3.89								
Makowska et al., 2016	PL	1	$2x10^6$	NA		CAS			I	8.66		4.88	4.41		3.00			4.36	3.01		0.70	0.62		1.60			1.04
									E	5.65		4.18	3.79		1.40			3.32								
Wen et al., 2016 [a]	CN	1	NA	GC	PC	A^2/O	SC	UV	I	8.60		5.75	6.94	6.42			4.01	6.82	1.74		1.96	1.19	2.67			1.53	1.83
									E	6.85		3.79	5.76	3.75			2.49	4.99								
		2	NA	GC	PC	A/O	SC	Chlorin	I	8.31		5.45	6.74	6.44			3.27	6.50	0.98		1.11	0.98	2.10			1.49	1.00
									E	7.33		4.33	5.76	4.33			1.78	5.50								
		3	NA	GC	PC	CASS	SC	UV	I	8.33		5.99	7.32	6.92			2.56	6.52	1.44		1.21	0.99	3.24			0.63	0.96
									E	6.88		4.78	6.33	3.68			1.93	5.57								
		4	NA	Screens + GC	PC	CASS	SC	UV	I	8.60		5.59	6.94	6.98			3.74	6.57	1.02		0.88	0.90	1.63			1.04	0.89
									E	7.57		4.71	6.04	5.35			2.70	5.66								
Rodriguez-Mozaz et al., 2015 [b]	ES	1	$1x10^5$	GC	PC	CAS	SC	None	I	6.48	6.00	5.70		6.00		5.00			2.57	3.00	2.00		3.30		1.30	
									E	3.90	3.00	3.70		2.70		3.70										
Laht et al., 2014	FI	2	$8x10^5$		PC	CAS	SC	P+ M + B	I	10.70		8.30	9.00		8.60		6.78		0.70		2.00	1.70		2.00		2.30	
									E	10.00		6.30	7.30		6.60		4.48									
	EE	3	$5x10^5$		PC	CAS	SC	P + M + B	I	10.00		8.48	8.90		9.00		6.60		2.00		1.18	1.90		1.40		1.30	
									E	8.00		7.30	7.00		7.60		5.30									
		4	$1x10^5$		PC	CAS	SC	P + M + B	I	10.30		8.60	9.00		9.00		6.70		0.60		1.00	1.70		1.40		1.40	
									E	9.70		7.60	7.30		7.60		5.30									
Czekalski et al., 2014 [a]	CH	1	$1x10^5$	Screens	PC	CAS	SC	None	I	8.34		7.00	6.29	5.27	6.15				0.74		0.51	0.74	0.55	0.67			
									E	7.60		6.49	5.55	4.72	5.48												

Table S2.4. Relative abundance of ARGs and MGEs to 16S rRNA in influent and effluent samples of 62 Dutch WWTPs: Values are expressed in $\log_{10}$. Values were obtained from averaging the log relative abundances of each gene in each sample. Relative abundance was obtained in each case with the following formula: relative abundance = $\log_{10}$ (ARG/ 16S rRNA). Acronyms: Inf: Influent; Eff: effluent; SD: Standard Deviation

Gene	*ermB*		*sul 1*		*Sul2*		*qnrS*		*tetM*		bla_{ctxM}		*intI1*		*korB*	
Sample	Inf	Eff	Inf	Eff	Inf	Eff	Inf	Eff	Inf	Eff	Inf	Eff	Inf	Eff	Inf	Eff
Average (Log_{10})	-2.04	-2.93	-1.90	-1.97	-2.81	-3.06	-2.85	-3.62	-3.08	-3.97	-4.12	-5.10	-1.99	-2.04	-3.01	-2.15
SD	0.31	0.44	0.21	0.27	0.18	0.27	0.26	0.43	0.36	0.48	0.26	0.67	0.23	0.25	0.38	0.26

Table S2.5. Effect of operational parameters on the reduction of ARGs and MGEs (univariate models, including *korB*). Models give the effect of single operational parameters adjusted for the hydraulic load factor and its interaction with the gene identity. Acronyms: n: number of plants with available information for that parameter; beta: model estimate. SE: standard error of the estimate. z: z statistics. p: p-value NP: no primary clarification; P: primary clarification without recirculation reject water; PR: Primary clarification with recirculation of reject water; B: biological phosphorus (P) removal; C: Chemical P removal; BC: Biochemical P removal. HRT: Hydraulic Retention Time; SRT: Sludge retention time; ACT: Anaerobic Contact Time.

			n	beta	SE	z	p
Factor	hydraulic load factor		62	-0.38	0.17	-2.21	0.03
	Size P.E. (150 g TOD)		62	-1.32E-07	3.66E-07	-0.36	0.72
	Primary settling		62				0.48
		NP	44				
		P	10	-0.14	0.17	-0.86	0.39
		PR	8	-0.17	0.18	-0.94	0.35
	P removal	none	23				0.67
		B	18	0.09	0.15	1.17	0.53
		C	7	0.24	0.21	0.42	0.25
		BC	14	0.07	0.16	-2.76	0.68
	HRT [hours]		45	0.01	0.01	1.42	0.16
	SRT [hours]		51	0.01	0.01	0.95	0.35
	ACT [hours]		11	0.14	0.14	0.41	0.33
	Effluent TSS [mg L^{-1}]		62	-0.02	0.01	-1.61	0.11

Table S2.6. Effect of operational parameters on the reduction of ARGs and MGEs (including *korB*) – results of multivariate modeling. Estimates of the effects of explanatory variables on gene removal (on $\log_{10}$ scale) during WWTP treatment (p values shown between brackets in italic; significant estimates are shown in bold) for the 7 best models that were of nearly identical quality as determined by AICc. For each model, both the results based on the subset of 37 plants for which all parameters were known and the results for the larger subset of plants for which these specific model parameters were known are given. The number of plants included in the model is shown under "n". Gene identity was also included in all models, as was the interaction between the Hydraulic Load Factor and the gene identity. For this interaction genes for which significant interactions with the Hydraulic Load Factor were found are listed (for *ermB*, *qnrS*, and *tetM*, the decrease in log removal with increasing Hydraulic Load Factor was more pronounced than for 16S rRNA). Acronyms: n: number of plants in the model; p: p-value NP: no primary clarification; P: primary clarification without recirculation of reject water; PR: Primary clarification with recirculation of reject water; N: not included in the model.

Inter-cept	Hydraulic Load Factor	E.TSS	HRT	Primary clarifier	SRT	Interaction between Hydraulic Load Factor : gene	AICc	*n*
2.07	-0.38 *(p=0.07)*	N	N	N	N	*ermB, qnrS, tetM*	270.3	37
2.05	-0.38 *(p=0.029)*	N	N	N	N	*ermB, qnrS, tetM*		62
1.98	-0.37 *(p=0.07)*	N	0.01 *(p=0.18)*	N	N	*ermB, qnrS, tetM*	270.6	37
1.97	-0.36 *(p=0.08)*	N	0.01 *(p=0.16)*	N	N	*ermB, qnrS, tetM*		45
2.18	-0.43 *(p=0.039)*	N	N	P: -0.31 *(p =0.13)* PR: -0.64 *(p=0.17)*	N	*ermB, qnrS, tetM*	270.8	37
2.10	-0.38 *(p= 0.03)*	N	N	P: -0.14 *(p=0.39)* PR: -0.17 *(p=0.35)*	N	*ermB, qnrS, tetM*		62
2.19	-0.39 *(p=0.06)*	-0.01 *(p=0.25)*	N	N	N	*ermB, qnrS, tetM*	271.2	37
2.20	-0.40 *(p=0.02)*	-0.02 *(p=0.11)*	N	N	N	*ermB, qnrS, tetM*		62
2.09	-0.38 *(p=0.07)*	-0.01 *(p=0.31)*	0.01 *(p=0.22)*	N	N	*ermB, qnrS, tetM*	271.8	37
2.10	-0.36 *(p=0.08)*	-0.01 *(p=0.22)*	0.01 *(p=0.21)*	N	N	*ermB, qnrS, tetM*		45
2.10	-0.42 *(p=0.045)*	N	0.009 *(p=0.33)*	P: -0.27 *(p=0.21)* PR: -0.59 *(p=0.22)*	N	*ermB, qnrS, tetM*	272.1	37
2.02	-0.38 *(p=0.07)*	N	0.01 *(p=0.24)*	P: -0.08 *(p= 0.69)* PR: -0.29 *(p=0.41)*	N	*ermB, qnrS, tetM*		45
1.94	-0.36 *(p=0.09)*	N	N	N	0.01 *(p=0.59)*	*ermB, qnrS, tetM*	272.3	37
1.91	-0.38 *(p=0.039)*	N	N	N	0.01 *(p=0.35)*	*ermB, qnrS, tetM*		51

Table S2.7. Final model of the removal of 16S, ARGs, and MGE in Dutch WWTP. Acronyms: beta: model estimate. SE: standard error of the estimate. z: z statistics. p: p-value. HLF: hydraulic load factor. The model is based on 496 observations of 8 genes in 62 WWTP. Pseudo-R^2 of the fixed effects is 0.54, pseudo-R^2 of the overall model is 0.86.

	beta	SE	z value	p
intercept	2.05	0.15	13.48	0.00
hydraulic load factor (HLF)	-0.38	0.17	-2.20	0.03
ermB	1.18	0.12	10.01	0.00
sul1	0.14	0.12	1.15	0.25
sul2	0.30	0.12	2.54	0.01
qnrS	1.14	0.12	9.63	0.00
tetM	1.12	0.12	9.52	0.00
intI	0.04	0.12	0.38	0.71
korB	-0.90	0.12	-7.65	0.00
interaction HLF - *ermB*	-0.36	0.13	-2.76	0.01
interaction HLF - *sul1*	-0.09	0.13	-0.66	0.51
interaction HLF - *sul2*	-0.06	0.13	-0.48	0.63
interaction HLF - *qnrS*	-0.31	0.13	-2.33	0.02
interaction HLF - *tetM*	-0.45	0.13	-3.37	0.00
interaction HLF - *intI1*	0.01	0.13	0.05	0.96
interaction HLF - *korB*	0.09	0.13	0.65	0.52

2.7. References

Bahl, M.I., Burmølle, M., Meisner, A., Hansen, L.H., Sørensen, S.J., 2009. All IncP-1 plasmid subgroups, including the novel e subgroup, are prevalent in the influent of a Danish wastewater treatment plant. Plasmid 62, 134–139. https://doi.org/10.1016/j.plasmid.2009.05.004

Baquero, F., Martínez, J.-L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19, 260–265. https://doi.org/10.1016/j.copbio.2008.05.006

Baran, W., Adamek, E., Ziemiańska, J., Sobczak, A., 2011. Effects of the presence of sulfonamides in the environment and their influence on human health. J. Hazard. Mater. 196, 1–15. https://doi.org/10.1016/j.jhazmat.2011.08.082

Barraud, O., Baclet, M.C., Denis, F., Ploy, M.C., 2010. Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. J. Antimicrob. Chemother. 65, 1642–1645. https://doi.org/10.1093/jac/dkq167

Barton, K., 2018. MuMIn: Multi-Model Inference. R package version 1.40.4.

Bates, D., Mächler, M., Bolker, B.M., Walker, S.C., 2016. Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Softw. 33, 251–264. https://doi.org/10.18637/jss.v067.i01

Bellanger, X., Guilloteau, H., Breuil, B., Merlin, C., 2014. Natural microbial communities supporting the transfer of the IncP-1β plasmid pB10 exhibit a higher initial content of plasmids from the same incompatibility group. Front. Microbiol. 5, 1–8. https://doi.org/10.3389/fmicb.2014.00637

Ben, W., Wang, J., Cao, R., Yang, M., Zhang, Y., Qiang, Z., 2017. Distribution of antibiotic resistance in the effluents of ten municipal wastewater treatment plants in China and the effect of treatment processes. Chemosphere 172, 392–398. https://doi.org/10.1016/j.chemosphere.2017.01.041

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439

Bradford, P.A., 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 14, 933–51, table of contents. https://doi.org/10.1128/CMR.14.4.933-951.2001

Brenciani, A., Bacciaglia, A., Vecchi, M., Vitali, L.A., Varaldo, P.E., Giovanetti, E., 2007. Genetic elements carrying erm(B) in Streptococcus pyogenes and association with tet(M) tetracycline resistance rene. Antimicrob. Agents Chemother. 51, 1209–1216. https://doi.org/10.1128/AAC.01484-06

Buelow, E., Bayjanov, J.R., Majoor, E., Willems, R.J., Bonten, M.J., Schmitt, H., van Schaik, W., 2018. Limited influence of hospital wastewater on the microbiome and resistome of wastewater in a community sewerage system. FEMS Microbiol. Ecol. 94. https://doi.org/10.1093/femsec/fiy087

Cai, L., Ju, F., Zhang, T., 2014. Tracking human sewage microbiome in a municipal wastewater treatment plant. Appl. Microbiol. Biotechnol. 98, 3317–3326. https://doi.org/10.1007/s00253-013-5402-z

Capodaglio, A.G., 2007. Improving sewage treatment plant performance in wet weather, in: Enhancing Urban Environment by Environmental Upgrading and Restoration. Kluwer Academic Publishers, Dordrecht, pp. 175–185. https://doi.org/10.1007/1-4020-2694-3_15

Council of the European Communities, 1991. Directive concerning the collection, treatment and discharge of urban wastewater and the discharge of wastewater from certain industrial sectors (91/271/EEC), Off J Eur Communities Ser L.

Czekalski, N., Gascón Díez, E., Bürgmann, H., 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. ISME J. 8, 1381–1390. https://doi.org/10.1038/ismej.2014.8

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461. https://doi.org/10.1038/nature10388

Davies, J., Davies, D., 2010. Origins and Evolution of Antibiotic Resistance. Microbiol. Mol. Biol. Rev. 74, 417–433. https://doi.org/10.1128/MMBR.00016-10

De Sotto, R.B., Medriano, C.A.D., Salcedo, D.E., Lee, H., Cho, Y., Kim, S., 2016. Effects of solids retention time on the fate of tetracycline resistance in SBRs for the treatment of carcass leachate. J. Environ. Manage. 181, 298–303. https://doi.org/10.1016/j.jenvman.2016.06.046

Di Cesare, A., Eckert, E.M., D'Urso, S., Bertoni, R., Gillan, D.C., Wattiez, R., Corno, G., 2016. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. Water Res. 94, 208–214. https://doi.org/10.1016/j.watres.2016.02.049

Dormann, C.F., Calabrese, J.M., Guillera-Arroita, G., Matechou, E., Bahn, V., Bartoń, K., Beale, C.M., Ciuti, S., Elith, J., Gerstner, K., Guelat, J., Keil, P., Lahoz-Monfort, J.J., Pollock, L.J., Reineking, B., Roberts, D.R., Schröder, B., Thuiller, W., Warton, D.I., Wintle, B.A., Wood, S.N., Wüest, R.O., Hartig, F., 2018. Model averaging in ecology: a review of Bayesian, information-theoretic, and tactical approaches for predictive inference. Ecol. Monogr. 88, 485–504. https://doi.org/10.1002/ecm.1309

Dröge, M., Pühler, A., Selbitschka, W., 2000. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. Mol. Gen. Genet. 263, 471–482. https://doi.org/10.1007/s004380051191

EPA, 1995. Waste Water Treatment Manuals: Preliminary Treatment. Environmental Protection Agency, Ireland.

Eramo, A., Reyes, H.D., Fahrenfeld, N.L., 2017. Partitioning of antibiotic resistance genes and fecal indicators varies intra and inter-storm during combined sewer overflows. Front. Microbiol. 8, 1–13. https://doi.org/10.3389/fmicb.2017.02024

Gao, P., Munir, M., Xagoraraki, I., 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci. Total Environ. 421–422, 173–183. https://doi.org/10.1016/j.scitotenv.2012.01.061

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226

Jechalke, S., Dealtry, S., Smalla, K., Heuer, H., 2013. Quantification of IncP-1 plasmid prevalence in environmental samples. Appl. Environ. Microbiol. 79, 1410–3. https://doi.org/10.1128/AEM.03728-12

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945. https://doi.org/10.1038/ismej.2014.191

Knapp, C.W., Zhang, W., Sturm, B.S.M., Graham, D.W., 2010. Differential fate of erythromycin and beta-lactam resistance genes from swine lagoon waste under different aquatic conditions. Environ. Pollut. 158, 1506–1512. https://doi.org/10.1016/j.envpol.2009.12.020

Kümmerer, K., 2009. Antibiotics in the aquatic environment--a review--part II. Chemosphere 75, 435–41. https://doi.org/10.1016/j.chemosphere.2008.12.006

Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2017. lmerTest Package: Tests in Linear Mixed Effects Models. J. Stat. Softw. 82. https://doi.org/10.18637/jss.v082.i13

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., Kisand, V., 2014. Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. PLoS One 9, 1–8. https://doi.org/10.1371/journal.pone.0103705

Lane, D., 1991. 16S/23S rRNA sequencing, in: E, S., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, New York., pp. 115–175.

LaPara, T.M., Madson, M., Borchardt, S., Lang, K.S., Johnson, T.J., 2015. Multiple Discharges of Treated Municipal Wastewater Have a Small Effect on the Quantities of Numerous Antibiotic Resistance Determinants in the Upper Mississippi River. Environ. Sci. Technol. 49, 11509–11515. https://doi.org/10.1021/acs.est.5b02803

Lee, J., Jeon, J.H., Shin, J., Jang, H.M., Kim, S., Song, M.S., Kim, Y.M., 2017. Quantitative and qualitative changes in antibiotic resistance genes after passing through treatment processes in municipal wastewater treatment plants. Sci. Total Environ. 605–606, 906–914. https://doi.org/10.1016/j.scitotenv.2017.06.250

Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Hawkey, P.M., Murray, A.K., Ukoumunne, O.C., Gaze, W.H., 2018. Exposure to and colonisation by antibiotic-resistant E. coli in UK coastal water users: Environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). Environ. Int. 114, 326–333. https://doi.org/10.1016/j.envint.2017.11.003

Lorenzo, P., Adriana, A., Jessica, S., Carles, B., Marinella, F., Marta, L., Luis, B.J., Pierre, S., 2018. Antibiotic resistance in urban and hospital wastewaters and their impact on a receiving freshwater ecosystem. Chemosphere 206, 70–82. https://doi.org/10.1016/j.chemosphere.2018.04.163

Lucas, F.S., Therial, C., Gonçalves, A., Servais, P., Rocher, V., Mouchel, J.M., 2014. Variation of raw wastewater microbiological quality in dry and wet weather conditions. Environ. Sci. Pollut. Res. 21, 5318–5328. https://doi.org/10.1007/s11356-013-2361-y

Makowska, N., Koczura, R., Mokracka, J., 2016. Class 1 integrase, sulfonamide and tetracycline resistance genes in wastewater treatment plant and surface water. Chemosphere 144, 1665–1673. https://doi.org/10.1016/j.chemosphere.2015.10.044

Marti, E., Jofre, J., Balcazar, J.L., 2013. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment tlant. PLoS One 8, 1–8. https://doi.org/10.1371/journal.pone.0078906

Metcalf, W., Eddy, C., 2003. Metcalf and Eddy Wastewater Engineering: Treatment and Reuse, in: Wastewater Engineering: Treatment and Reuse McGraw Hill. New York, NY. https://doi.org/10.1016/0309-1708(80)90067-6

Mines, R.O., Lackey, L.W., Behrend, G.H., 2007. The impact of rainfall on flows and loadings at Georgia's wastewater treatment plants. Water. Air. Soil Pollut. 179, 135–157. https://doi.org/10.1007/s11270-006-9220-0

Moura, A., Henriques, I., Smalla, K., Correia, A., 2010. Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes. Res. Microbiol. 161, 58–66. https://doi.org/10.1016/j.resmic.2009.11.004

Munir, M., Wong, K., Xagoraraki, I., 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Res. 45, 681–693. https://doi.org/10.1016/j.watres.2010.08.033

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700. https://doi.org/0099-2240/93/030695-06$02.00/0

Nethmap/Maran, 2018. Melles, DC Mouton, JW. National Institute for Public Health and the Environment. https://doi.org/10.21945/RIVM-2018-0046

Neyestani, M., Dickenson, E., McLain, J., Obergh, V., Quinones, O., Rock, C., Gerrity, D., 2017. Solids retention time, influent antibiotic concentrations, and temperature as selective pressures for antibiotic resistance in activated sludge systems. Environ. Sci. Water Res. Technol. 3, 883–896. https://doi.org/10.1039/c7ew00171a

Novo, A., André, S., Viana, P., Nunes, O.C., Manaia, C.M., 2013. Antibiotic resistance, Antimicrobial residues and bacterial community composition in urban wastewater. Water Res. 47, 1875–1887. https://doi.org/10.1016/j.watres.2013.01.010

Park, A.K., Kim, H., Jin, H.J., 2010. Phylogenetic analysis of rRNA methyltransferases, Erm and KsgA, as related to antibiotic resistance. FEMS Microbiol. Lett. 309, 151–162. https://doi.org/10.1111/j.1574-6968.2010.02031.x

Peak, N., Knapp, C.W., Yang, R.K., Hanfelt, M.M., Smith, M.S., Aga, D.S., Graham, D.W., 2007. Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. Environ. Microbiol. 9, 143–151. https://doi.org/10.1111/j.1462-2920.2006.01123.x

Pei, R., Kim, S.-C., Carlson, K.H., Pruden, A., 2006. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res. 40, 2427–2435. https://doi.org/10.1016/j.watres.2006.04.017

Puig, S., van Loosdrecht, M.C.M., Flameling, A.G., Colprim, J., Meijer, S.C.F., 2010. The effect of primary sedimentation on full-scale WWTP nutrient removal performance. Water Res. 44, 3375–3384. https://doi.org/10.1016/j.watres.2010.03.024

R Core Team, 2018. R: A language and environment for statistical computing. R Found. Stat. Comput. Vienna.

Rafraf, I.D., Lekunberri, I., Sànchez-Melsió, A., Aouni, M., Borrego, C.M., Balcázar, J.L., 2016. Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. Environ. Pollut. 219, 353–358. https://doi.org/10.1016/j.envpol.2016.10.062

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242. https://doi.org/10.1016/j.watres.2014.11.021

Rouleau, S., Lessard, P., Bellefleur, D., 1997. Behaviour of a small wastewater treatment plant during rain events. Can. J. Civ. Eng. 24, 790–798. https://doi.org/10.1139/l97-035

Sabri, N.A., Schmitt, H., Van der Zaan, B., Gerritsen, H., Zuidema, T., Rijnaarts, H.H.., Langenhoff, A.A.M., 2018. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. J. Environ. Chem. Eng. https://doi.org/10.1016/j.jece.2018.03.004

Schmitt, H., 2017. Bronnen va antibioticaresistentie in het milieu en mogelijke maatregelen. https://doi.org/10.21945/RIVM-2017-0058

Schmitt, H., ter Laak, T., Karen, D., 2016. Development and dissemination of antibiotic resistance in the environment under environmentally relevant concentrations of antibiotics and its risk assessment Literature Study.

Sheppard, A.E., Stoesser, N., Wilson, D.J., Sebra, R., Kasarskis, A., Anson, L.W., Giess, A., Pankhurst, L.J., Vaughan, A., Grim, C.J., Cox, H.L., Yeh, A.J., Sifri, C.D., Walker, A.S., Peto, T.E., Crook, D.W., Mathers, A.J., 2016. Nested Russian doll-like genetic mobility drives rapid dissemination of the carbapenem resistance gene blakpc. Antimicrob. Agents Chemother. 60, 3767–3778. https://doi.org/10.1128/AAC.00464-16

Smith, R., Elger, S., Mleziva, S., 2014. Wastewater: Solids retention time control in wastewater treatment. Filtr. Sep. 52, 12–17. https://doi.org/10.1016/S0015-1882(14)70102-8

Tsutsui, H., Anami, Y., Matsuda, M., Inoue, D., Sei, K., Soda, S., Ike, M., 2010. Transfer of plasmid pJP4 from Escherichia coli and Pseudomonas putida to bacteria in activated sludge developed under different sludge retention times. J. Biosci. Bioeng. 110, 684–9. https://doi.org/10.1016/j.jbiosc.2010.07.009

Verburg, I., García-Cobos, S., Leal, L.H., Waar, K., Friedrich, A.W., Schmitt, H., 2019. Abundance and antimicrobial resistance of three bacterial species along a complete wastewater pathway. Microorganisms 7. https://doi.org/10.3390/microorganisms7090312

Wei, T., Simko, V., 2016. The corrplot package. CRAN Repos.

Weissbrodt, D.G., Shani, N., Holliger, C., 2014. Linking bacterial population dynamics and nutrient removal in the granular sludge biofilm ecosystem engineered for wastewater treatment. FEMS Microbiol. Ecol. 88, 579–595. https://doi.org/10.1111/1574-6941.12326

Wen, Q., Yang, L., Duan, R., Chen, Z., 2016. Monitoring and evaluation of antibiotic resistance genes in four municipal wastewater treatment plants in Harbin, Northeast China. Environ. Pollut. 212, 34–40. https://doi.org/10.1016/j.envpol.2016.01.043

CHAPTER 3

3

Annual dynamics of antimicrobials and resistance determinants in flocculent and aerobic granular sludge treatment systems

Abstract

The occurrence and removal patterns of 24 antimicrobial agents and antibiotic resistant determinants, namely 6 antibiotic resistance genes (ARGs) and 2 mobile genetic elements (MGEs), and the fecal indicator *E. coli* were investigated in three full-scale wastewater treatment plants. Their waterlines and biosolids lines (including secondary treatment based on both granular and activated sludge) were sampled monthly throughout one year. Samples were analyzed by means of LC-MS/MS, qPCR and cell enumeration, respectively. The influence of rainfall, temperature and turbidity on the occurrence and removal of the aforementioned agents was assessed through statistical linear mixed models. Ten antimicrobial agents (macrolides, fluoroquinolones, tetracyclines, and sulphonamides) were commonly found in influent concentrations of 0.1-2 μg L^{-1}, and the predominant ARGs were *ermB* and *sul1* (6.4 and 5.9 log_{10} mL^{-1} respectively). Warmer temperatures slightly reduced gene concentrations in influent whilst increasing that of *E. coli* and produced an uneven effect on the antimicrobial concentrations across plants. Rainfall diluted both *E. coli* (-0.25 logs, $p<0.001$) and antimicrobials but not genes. The wastewater treatment reduced the absolute abundance of both genes (1.86 logs on average) and *E. coli* (2.31 logs on average). The antimicrobials agents were also partly removed, but 9 of them were still detectable after treatment, and 6 accumulated in the biosolids. ARGs were also found in biosolids with patterns resembling those of influent. No significant differences in the removal of antimicrobials, genes and *E. coli* were observed when comparing conventional activated sludge with aerobic granular sludge. Irrespective of the type of sludge treatment, the removal of genes was significantly reduced with increasing hydraulic loads caused by rainfall (-0.35 logs per Δaverage daily flow $p<0.01$), and slightly decreased with increasing turbidity. (-0.02 logs per 1Δ nephelometric turbidity unit $p < 0.05$) .

A modified version of this chapter has been published as: Pallares-Vega, R., Hernandez Leal, L., Fletcher, B.N., Vias-Torres, E., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2021. Annual dynamics of antimicrobials and resistance determinants in flocculent and aerobic granular sludge treatment systems. Water Res. 190, 116752. https://doi.org/10.1016/j.watres.2020.116752

3.1. Introduction

The occurrence of anthropogenic antibiotic resistance genes (ARGs) in the environment seems to be strongly related to fecal pollution (Karkman et al., 2019). Human fecal bacteria are transported through sewage networks and wastewater treatment plants (WWTPs) to the environment. WWTPs, primarily designed for nutrient removal, have therefore been hypothesized as key vectors in the environmental dissemination of antibiotic resistance (Rizzo et al., 2013). ARG fate in WWTPs has been assessed in recent years, providing a wide range of results. In some cases, the WWTPs reduced the absolute concentration of ARGs (Di Cesare et al., 2016; Rodriguez-Mozaz et al., 2015), while in others, the relative and even absolute abundances of ARGs increased after treatment (Rafraf et al., 2016).

There is a growing interest in determining which technologies or operational conditions achieve greater ARGs removal. However, due to the wide diversity of treatment processes, it is difficult to obtain general results that are applicable across the variety of WWTP systems and ARG types (Korzeniewska and Harnisz, 2018; Novo and Manaia, 2010). Previously, broad sampling efforts to analyze the influent and effluent of more than 60 WWTPs in The Netherlands helped to determine that the removal of ARGs was significantly smaller when the baseline hydraulic load of the WWTPs increased because of rainfall events (Pallares-Vega et al., 2019). Yet, process design and operational parameters did not explain the remaining variability even under dry weather conditions. This suggests that other factors contributed to the detected differences.

One factor that may impact the occurrence and removal of ARGs is the presence of antimicrobial and disinfectant residues. These compounds are collected in the wastewater along with the feces and might accumulate in the sewage sludge (Gao et al., 2012; Göbel et al., 2005). Antimicrobial and disinfectant residues may enhance the co-selection of resistance genes and promote horizontal gene transfer even at subtherapeutic concentrations (Gullberg et al., 2014). In addition, fluctuations in other abiotic factors such as temperature might also influence the occurrence and removal of ARGs. Higher antimicrobial consumption in colder seasons (Coutu et al., 2013; Marx et al., 2015) might increase the discharge of resistant bacteria and their ARGs into the sewer. In addition, cold temperatures reduce the efficiency of water treatment (Johnston et al., 2019), and might also reduce the ability to remove ARGs. Literature addressing the effect of temperature on the occurrence and fate of ARGs during wastewater treatment is, however, scarce. A few studies have focused on quantifying the

seasonal variation of ARGs in influent and their removal (Caucci et al., 2016; Jiao et al., 2018). Moreover, temperature or seasonality have seldom been addressed in combination with rainfall or flow (Schages et al., 2020). Thus, there is a need for a comprehensive approach to studying the combined effect of these variables on ARG patterns.

Furthermore, biotic factors such as the accumulation of ARGs in the natural microbiome of the biological treatment deserve attention. In the biological treatment, the microbial community, which converts wastewater compounds, is aggregated into flocs (activated sludge), biofilms, or granular sludge. Such bio aggregates might provide a suitable environment for cell-to-cell interactions and genetic exchange of mobile genetic elements (MGE) containing ARGs, (Manaia et al., 2018) potentially increasing the so-called resistome of the sludge. Diverse sludge types (flocculent, granules) might accumulate ARGs differently, derived from their characteristic physical structure and microbial community. Ultimately, the intrinsic sludge resistome may counterbalance a further elimination of ARGs during wastewater treatment. The accumulation of ARGs in the biosolids is also of concern, given that sludge is often processed and applied as a fertilizer due to its high content of organic nutrients and phosphorus. As such, this practice comprises another possible route for the spread of anthropogenic ARGs to the environment (Rahube et al., 2014).

Generally, studies covering the effect of a broad number of variables on ARGs are restricted to few time-point measurements. Although cross-sectional studies provide relevant information, it is necessary to investigate whether measurements at single time points are valid throughout extended periods. Such information is needed to determine suitable sampling strategies to answer specific research questions. Hence, the aim of the present study was to investigate the occurrence and fate of ARGs, MGEs, and viable fecal bacteria over one year of operation at three different full-scale municipal WWTPs located in The Netherlands. These treatment plants performed biological nutrient removal, with three systems based on activated sludge and one system based on aerobic granular sludge. Besides gene determinants, we investigated the presence of selective agents as antimicrobial compounds and disinfectants in both the water and biosolids lines. Analyses were aimed at determining the role of abiotic parameters such as the Hydraulic Load Factor, seasonal temperature, and the effluent turbidity (as a surrogate for effluent TSS) on the gene removal capacity of WWTPs for an extended sampling period of one year and to study the degree of temporal variability.

3.2. Material and Methods

3.2.1. Characteristics of the selected WWTPs

Three Dutch WWTPs (**supplementary information Figure S3.1**) of different sizes and process designs, were considered. The sampling points are displayed in **Figure 3.1**.

WWTP1 (Leeuwarden, 226'667 p.e.) processes 25'000 m^3 d^{-1} under dry weather conditions. The biological nutrient removal activated sludge process is operated on raw wastewater. To support the biological phosphate removal, iron (FeII) is added to the activated sludge tanks. The chemical oxygen demand load (COD) to the plant consists of 56% household wastewater and 26% industrial wastewater (from which half corresponds to a dairy industry). The catchment area includes a medium-size hospital (650 beds) with a load contribution of 1%.

WWTP2 (Harnaschpolder, Den Haag, 1'260'000 p.e) is the largest plant in The Netherlands. It treats an average of 150'000 m^3 d^{-1}. 84% of the COD load comes from households and 16% from industry. The catchment area includes several hospitals, totalling 2610 beds. The design of this WWTP consists of 8 identical parallel lines. Each line is composed of primary settling and a biological nutrient removal activated sludge process.

WWTP3 (Garmerwolde, Groningen, 340'146 p.e.) treats 71'800 m^3 d^{-1} (64% households,14% industry, and 1% hospital (totalling 1920 beds) in two separate treatment lines. Approximately 50% of the influent is treated in a two-stage activated sludge adsorption-bioxidation (AB) process (Bönke, 1977). The other half of the wastewater is treated by an aerobic granular sludge process (Nereda®). The AB system has been described in detail by De Graaff et al. (2016). The raw influent undergoes two consecutive treatment steps. First, the organic content is removed in the highly loaded A-stage activated sludge process operated at a short solid retention time. Phosphate is removed in the A-stage by the addition of iron (Fe III). After the intermittent clarifier, the second B-stage activated sludge process is operated at a long solids retention time to allow nitrification and removal of the remaining biological oxygen demand (BOD). Nitrogen removal is limited by the low BOD content; therefore, methanol is added to promote denitrification. The parallel aerobic granular sludge line (Pronk et al., 2015) includes a buffer tank to store the influent wastewater for up to 3 h before treatment, and two Nereda® reactors containing aerobic granular sludge. The activated sludge is mainly in granular form but also contains a fraction of flocs (Ali et al., 2019). The HRT of this reactor is 6-h in dry weather conditions. All removal processes (BOD, denitrification, and P removal) occur under the alternation of anaerobic feeding and aeration. Denitrification partly occurs as simultaneous

nitrification/denitrification and partly by on/off aeration. Phosphate removal is essentially biological, with supplemental addition of iron (Fe III) only taking place during extreme rain weather flow.

In all three WWTPs, the surplus sludge is digested in a mesophilic reactor, and the digested sludge is subsequently dewatered and incinerated. In WWTP1, the digester receives sludge from other industrial physical-chemical treatment plants in the area.

3.2.2. Collection of samples from the wastewater line and the biosolids line

Waterline and biosolids line samples were taken every month over one year from April 2017 until March 2018 from the three aforementioned WWTPs.

For the waterline, volumes of 1 L of 24-h flow-proportional composite samples were collected in sterile plastic bottles (VWR, NL), except for the effluent of the AB line in WWTP3 that lacked an autosampler. In that case, a grab sample was collected instead. All autosamplers for the waterline collection had a refrigeration system to ensure samples were kept cold during the 24-h collection period. To account for possible daily variations, the waterline samples were collected three days in a row except for WWTP3, for which this was not possible due to technical reasons, and only one sample per month was collected. All waterline samples were filtered for DNA and culture-based methods within 6 h after collection. Filters were stored at -20ºC upon DNA extraction. Waterline samples were backed up frozen at -20ºC for downstream chemical analysis.

For the biosolids line, samples were collected once per month as grab samples in 0.5 L sterile plastic bottles. The flocculent biomass samples (further referred to as “AS”) were collected from the mixed liquors from the activated sludge tanks. The aerobic granular sludge samples (further referred to as “AGS”) were taken from the purge of excess sludge. Digested sludge samples (further referred to as “DS”) were taken as a grab sample from the digested sludge leaving the digestor. All samples were stored in cooling boxes and kept cold (4ºC) during transportation. Biosolids line samples were serially diluted and filtered for the culture-based method within ~6 h after collection. Additionally, aliquots were backed up frozen at -20ºC until processing for the downstream chemical analysis and DNA extraction.

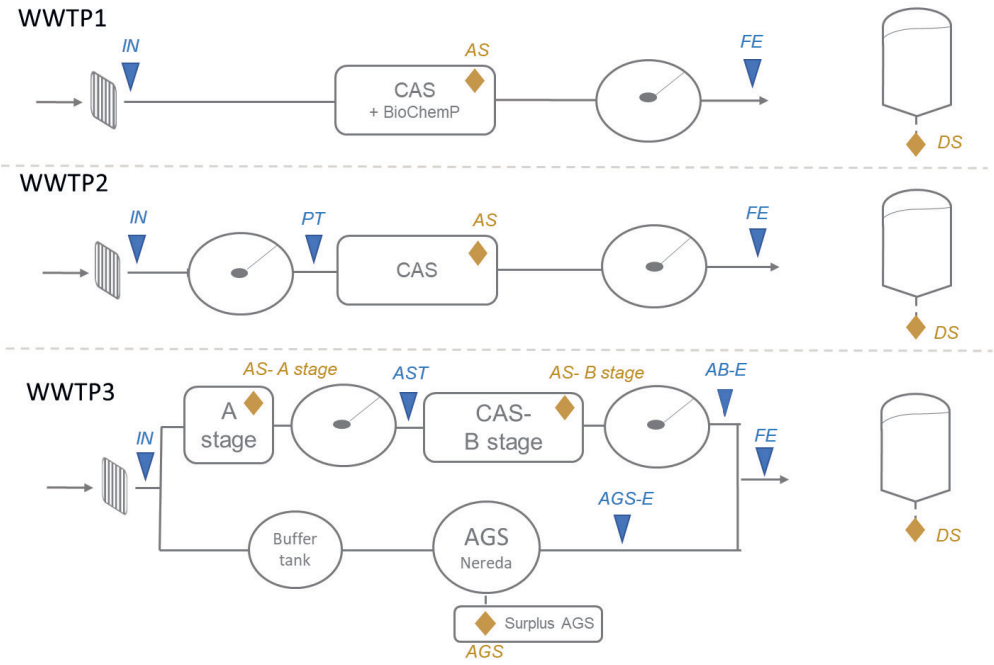


Figure 3.1. Plant design and sampling points (in italic) of the waterline (▼) and the biosolids line (◆) of the 3 WWTPs included in this study. Abbreviations: AGS: Aerobic Granular Sludge; CAS: Conventional Activated Sludge, BioChemP: BioChemical Phosphorus Removal. Samples abbreviations: IN: Influent; PT: after Primary Treatment; AST: After A stage; AB-E: Effluent after AB treatment; AGS-E: Effluent after Aerobic Granular Sludge treatment; FE: Final Effluent; DS: Digested Sludge

3.2.3. Filtration for E. coli enumeration

Monitoring the presence and removal of *E. coli* was used to evaluate whether fecal bacteria and ARGs would follow similar trends. *E. coli* was chosen as a surrogate for fecal indicators. For the waterline, samples were processed as previously described in Verburg et al. (2019). For the biosolids line, 2 g of homogenous sample were re-suspended in 20 mL of the saline solution (NaCl) 0.85% (w/v). Serial dilutions were performed and filtered as indicated for the waterline.

After filtration, the resulting filters from both the water and the biosolids lines were plated on Tryptone Bile X-Glucuronide (TBX) selective media (Oxoid, Thermofisher, UK). The plates were incubated for 24 h at 37ºC, and The CFUs were enumerated following ISO guidelines (ISO 8199:2005-12).

3.2.4. DNA extraction and qPCR analysis.

In order to analyze the water and sludge samples for the presence of ARGs and MGEs, samples were pre-treated and extracted as follows.

For the waterline samples, volumes of 200 mL of effluent and 25 mL of influent (and samples with similar solids content) were filtered through 0.22 µm Durapore PVDF membranes (Merck-Millipore) in a Millipore-Sigma filtration system. The filters were frozen at -20°C upon extraction. The DNA extraction for waterline samples was performed using the DNeasy kit Power Water from (Qiagen, NL), following the manufacturer's instructions.

For the biosolids line samples, 0.50 g of all types of AS, 0.05 g of DS and 0.10 g of AGS samples were extracted according to the MiDAS Field Guide to the Microbes of Activated Sludge and Anaerobic Digesters, versions 7.0 (AS), and 1.0 (DS) (ref: http://www.midasfieldguide.org) with small modifications: the bead-beating step was performed at 6800 rpm in a Precellys homogenizer (Bertin Technologies SAS, FR) which is equivalent to a speed mode of 5.5 in the FastPrep homogenizer. The final elution was reduced to 100 µL to achieve more concentrated DNA extracts.

After the extraction, the DNA extracts were diluted 10 or 100-fold to avoid inhibition and ensure the target was within the range of quantification. The diluted DNA was analyzed by quantitative polymerase chain reaction (qPCR). The panel of studied genes (**Table 3.1**) included six ARGs (*sul1*, *sul2*, *tetM, qnrS*, *ermB,* and $bla_{CTX\text{-}M}$), two MGEs (*intI1* gene and *korB* gene). The 16S rRNA gene acted as a surrogate for total bacteria. The reactions were prepared and performed as previously described in Pallares-Vega et al. (2019) with small modifications for the *korB* assay. This information is specified in **section 3.6 (Appendix A**)

3.2.5. Total solids content and antimicrobial and disinfectant compounds in water and biosolids samples

The total solids (TS) content of the sludge samples was determined by standard methods (Clesceri et al., 1998). This information was required to express the results from the microbiological, molecular, and physicochemical analyses in biosolids as normalized to the total solids (TS).

The determination of antimicrobial and disinfectant residues in waterline and biosolids lines samples was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The analyzed compounds are compiled in **Table 3.1**. Pretreatment and analysis of the

waterline samples were performed, as described in Verburg et al. (2019). Biosolids sample preparation, specifications of the device, and run are summarized in **section 3.7 (Appendix B).** For each sample, a surrogate sample spiked with the known concentrations was used to calculate the recovery.

Table 3.1. Gene targets for qPCR and chemical targets for LC-MS/MS analysis of antimicrobial residues

qPCR targets					
Gene	**Function**	**Group**	**Gene**	**Resistance to**	**Group**
16S rRNA	Ribosomal sub unit	All bacteria	*ermB*	Erythromycin	ARGs
			sul1	Sulfonamides	
intI1	Integrase 1	MGE	*sul2*	Sulfonamides	
korB	(IncP-1 plasmids)		*tetM*	Tetracyclines	
			qnrS	Fluoroquinolones	
			bla_{CTX-M}	β-lactamases	
Antimicrobials and disinfectants					
Abrev.	**Compound**	**Class**	**Abrev.**	**Compound**	**Class**
DM	Dimetridazole	Azoles	LINCOM	Lincomycin	Lincosamides
AMOX	Amoxicillin	β-lactams	CM	Clindamycin	
AMP	Ampicillin		DOX	Doxycycline	Tetracyclines
PENG	Penicillin G		OTET	Oxytetracycline	
PENV	Penicillin V		TET	Tetracycline	
CFT	Cefotaxime	Cephalosporines	SMX	Sulfamethoxazole	Sulfonamides
AZI	Azithromycin	Macrolides	SUCLOP	Sulfachloropyridazine	&
CLAR	Clarithromycin		SUDOX	Sulfadoxine	Trimethoprim
ERY	Erythromycin		SUPY	Sulfapyiridine	
TYLOS	Tylosin		TRIM	Trimethoprim	
TILMIC	Tilmicosin		BAC_{12}	Benzalkonium chloride	Quaternary
CIP	Ciprofloxacin			12	ammonium
OFX	Ofloxacin	Fluoroquinolones	BAC_{14}	Benzalkonium chloride	compounds
FLUMEQ	Flumequine			14	(QACs)

3.2.6. Sampling factors and statistical analysis

Information about abiotic factors during sampling collection was obtained as follows: data about daily flow and average annual flows for 2017 and 2018 were obtained from the WWTP operators. With this data, the Hydraulic Load Factor (HLF) was calculated. This parameter stands for the ratio of the flow (*i.e.,* volume of water treated) on the day of sampling divided by the average daily flow (derived from the annual flow) of each WWTP (Pallares-Vega et al., 2019), as calculated in **Eq (2.1).**

Air temperature on the day of sampling was retrieved from https://weerstatistieken.nl. Turbidity in effluent samples was analyzed by means of a turbidimeter (2100 N IS, Hach). The influence of these factors in the incoming and removal of genes and *E. coli,* as well as the role

of intermediate steps in the removal of genes, were analyzed by linear models and linear mixed models, further described in detail in **section 3.8. (Appendix C)**

3.3. Results and Discussion

3.3.1. Antimicrobials, ARGs and E. coli in the influent: role of rain dilution and seasonal temperature

From the 23 antimicrobials included in this study, 10 were detected in the influent of the three WWTPs, in general, in concentrations within the ng L^{-1} scale (**supplementary information Table S1**). Overall, the values observed in this study were consistent with several other works across different countries (Felis et al., 2020). The fluoroquinolone ciprofloxacin was the most abundant compound in the influent, consistently exceeding the predicted no inhibitory concentrations (PNEC-MIC) described by (Bengtsson-Palme and Larsson, 2016) for selection of antimicrobial resistance for this compound (0.064 µg L^{-1}), with maximum levels reaching 1.20 µg L^{-1}. The macrolides azithromycin and clarithromycin and the therapeutic group of sulphonamides (sulphapyridine sulfamethoxazole)-trimethoprim were also commonly detected in influent and sometimes exceeding their corresponding PNEC-MIC levels. In contrast, tetracyclines (doxycycline and tetracycline), which are the second most consumed antibiotics, were prevalent in the influent but below their PNEC-MIC levels (2 and 1 ug $L^{-1,}$ respectively).

The quaternary ammonium compounds (QACs), benzalkonium chloride (BAC) 12, and 14 were also prevalent in the influent, especially in WWTP1, with values up to 8.22 µg L^{-1}. These QACs are used as surfactants in cleaning products and disinfectants. *Qac* resistance genes are frequently associated with class 1 integrons and other ARGs included in those MGEs (Gillings et al., 2009). QACs selective pressure might entail the co-selection of MGEs and their associated ARGs. Possibly, the use of disinfectants by a neighbouring dairy industry in the catchment area of WWTP1 (contributing to ~12% of the influent) could explain these higher levels.

The occurrence of a selected panel of ARGs and MGEs in the influent of the waterline followed similar trends across the three WWTPs (supplementary information Table S2). From the ARGs selected, *ermB* (6.39 log gene copies mL^{-1}*)* and *sul1* (5.85) had the highest average annual concentration values, while the β-lactamase bla_{CTX-M} had the lowest (4.05). The overall ARG patterns are in accordance with our previous study of more than 60 Dutch WWTPs (Pallares-Vega et al., 2019) and in other recent works (Di Cesare et al., 2016; Rodriguez-Mozaz et al., 2015). The high concentration of *ermB* gene in the influent cannot be associated with a

direct antibiotic selective pressure within sewage, as erythromycin residues were not detected in the influent. The high occurrence of *ermB* might result from its location in Lactobacillales, which are common in the gut microbiome and, therefore, predominant taxa in the influent (Ali et al., 2019; Cai et al., 2014). The high occurrence of *sul1* may be explained by its extended use in the past and its association with MGE, such as integron class 1 (clinical integron). Their presence could also be maintained by the persistence of sulphonamide antibiotic residues in wastewater (Baran et al., 2011). As for the prevalence of MGEs, both *korB* (standing for IncP-1 plasmids) and *intI1*, encoding for the integrase of class 1 integron, were ubiquitous in the influent samples. Moreover, *intI1* had a significantly higher concentration ($p< 0.01$) in the influent of WWTP1 (7.04 log gene copies mL^{-1}) when compared to the other two WWTPs (6.16 logs on average), and above the range measured in our previous study (Pallares-Vega et al., 2019). These results might be explained through co-selection events by the extended use of QACs within the dairy industry facilities. Further analysis addressing the presence of *qac* resistance genes and *qac-intI1* relation would be necessary to confirm such a hypothesis.

To investigate the role of rainfall on the occurrence of antimicrobials, ARGs, and *E. coli* in influent, both their concentrations and their daily loads per population equivalent (pe) (*i.e.* the absolute number of gene copies passing the WWTP per day divided by the population equivalent) were studied. Unlike concentrations, the daily load per pe should be constant despite differences in rainfall dilution if freshly discharged human feces was the only source of these compounds. Therefore, using daily loads or daily loads per pe (of both genes and *E. coli*) for graphical representation and model response should be better suited to detect possible temperature or season effects.

Increased rainfall led, as expected, to decreased concentrations of *E. coli* (-0.25 logs per Δaverage daily flow, $p<0.001$) but not to decreased daily loads per pe (+0.06 logs per Δaverage daily flow, $p=0.15$), **supplementary information Figure S3.2, Figure S3.3 and Table S3.3** models 5-7, confirming the dilution effect of *E. coli* upon heavier rainfall. In contrast, for the studied genes, the reduction in concentrations with increased rainfall was less clear (the best model did not include HLF as a determinant), and there was a significant positive effect of increased rainfall on the resistance gene when the daily loads per pe were used as the response variable (+0.42 logs per Δ average daily flow, $p<0.001$).

An increase of the daily loads per pe of ARGs and MGEs with increased rainfall might point to an additional source of genes besides freshly discharged feces. We hypothesized that

such a source could consist of resident antibiotic resistant microbiota in the sewers, located for instance, within sewer biofilms or sewer sediments (Auguet et al., 2017). These might also have been introduced initially into sewers with fecal microbiota. With increasing flow due to rainfall, a washout of the sewer microbiota could increase the incoming loads of resistance genes per pe, similar to washout of in-sewer stocks of, e.g. organic matter (Gromaire et al., 2001). The contrasting behaviour of the loads per pe of *E. coli* during rainfall events might indicate a minor accumulation of this organism in the sewer pipes. A limited accumulation of *E. coli* O157:H7 and gammaproteobacteria in sewer biofilm has been previously detected (Auguet et al., 2017). However, the observed discrepancies between genes and *E. coli* might also be a consequence of a methodology bias. Unlike qPCR, culturable-based methods account for neither the dead nor a viable-but not-culturable fraction of bacteria.

With respect to seasonal temperature, the studied agents in influent (antimicrobials, genes, and *E. coli*) showed an inconsistent response (**Figure 3.2, supplementary information Figure S3.2-Figure S3.4 and Table S3.3** models 1-7). The antibiotic loads varied per plant, with only WWTP2 showing an increase of antibiotic loads at colder temperatures. With respect to resistance genes, a slight but significant decrease of incoming genes (for both concentration and daily loads per pe) with increasing temperatures was observed for the set of genes as a whole (-0.02 logs per ºC, $p<0.05$). In contrast, increasing temperatures significantly enhanced both *E. coli* concentrations and daily loads per pe in the influent of all three WWTP. The effect was mild as per degree increased (+0.03 logs per ºC, $p <0.001$).

Overall, there is limited information regarding seasonal fluctuations in the influent of both antimicrobials and ARGs. Some studies have observed an increase in loads of some antibiotics during winter months (Coutu et al., 2013; Marx et al., 2015). This has been related to higher consumption of antibiotics used to treat winter-related conditions (Marx et al., 2015), which could increase the selective pressure and enhance the occurrence of resistant bacteria and ARGs in the sewage. This hypothesis is supported by three studies conducted in German and Chinese WWTPs. In these studies, either higher relative abundance of several ARGs (Caucci et al., 2016; Jiao et al., 2018) or higher absolute concentration of numerous *bla* genes (Schages et al., 2020) were found in cold seasons. In contrast, Karkman et al. (2016) did not observe any difference across seasons after quantifying the relative concentration of a broad set of ARGs in a Finnish WWTP. Differences in antibiotic prescriptions across countries and the degree of seasonality might explain the contradicting observations. Moreover, integrating flow variations of the sampling days into the studies might contribute to explain the uneven results.

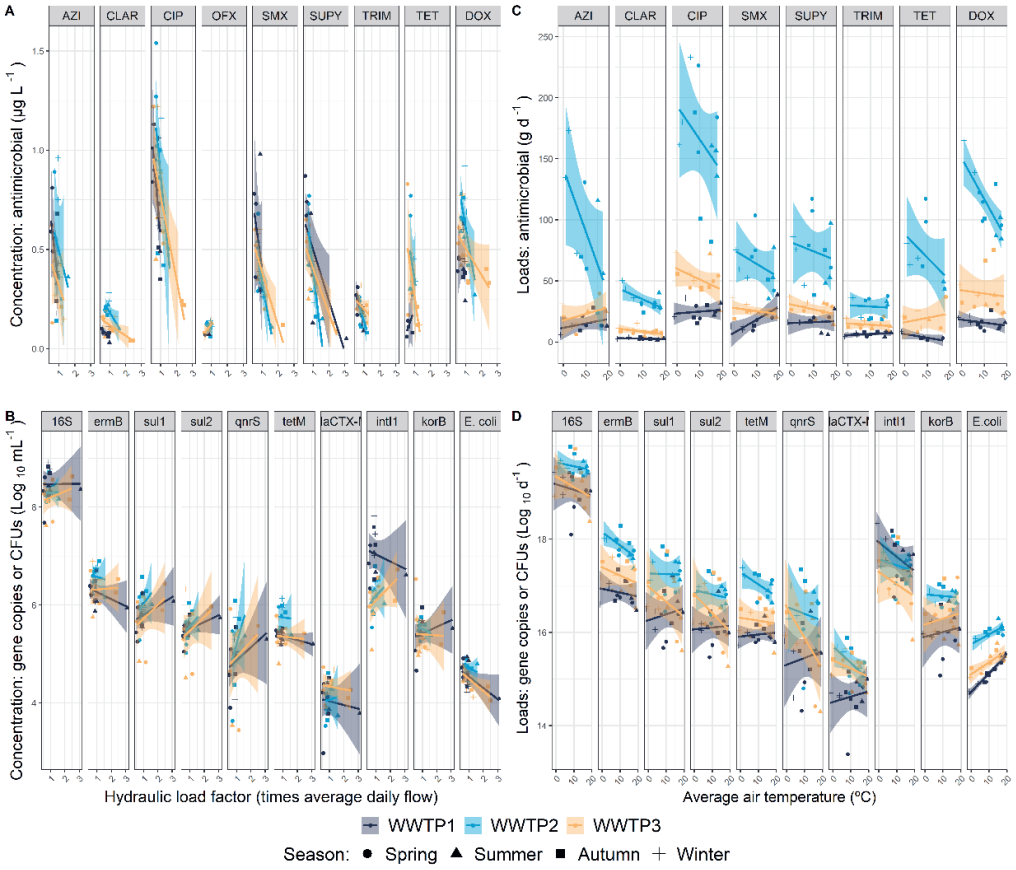


Figure 3.2. Incoming loads of antimicrobials (panel A) and genes and *E. coli* (panel B) in the function of the average atmospheric temperature on the day of sampling in three Dutch WWTPs. Loads are used instead of concentration to remove the influence of flow and graphically observe only the variability caused by seasonal changes in temperature. Values corresponding to each of the four seasons are displayed with different symbols.

3.3.2. Removal of resistance determinants and E. coli

3.3.2.1 Removal of E. coli and gene determinants through conventional water treatment and aerobic granular sludge

All three WWTPs significantly ($p<0.001$) removed the fecal indicator bacteria and the tested genes. The removal efficiency varied across WWTPs and measured agent **(Figure S3.3**, **supplementary information Table S3.3**). WWTP1 achieved the best removal for both *E. coli* and genes, with an average removal of 2.31 logs for *E. coli* (**supplementary information Figure S3.5**) and ≥2 logs of tested genes (except for *korB*). The other two WWTPs performed significantly worse (+ 0.4 logs $p<0.001$) removing both ARGs and *E. coli* (+ 0.2 - +0.4 $p<0.05$, **supplementary information Table S3.3**, models 11 and 12), although within the range of removal previously observed for Dutch WWTPs (Pallares-Vega et al., 2019). Moreover, the patterns for

gene removals were, in general, similar in all three WWTPs and are following our previous study. The most successfully removed genes were *ermB* (2-3 logs on average), *tetM*, and bla_{CTX-M} (2 logs on average). Bla_{CTX-M} was undetectable or unquantifiable in 10-40% of the effluent samples of WWTPs 3 and 1, respectively. The most resilient ARGs of the panel were those relating to sulphonamides (*sul1* and *sul2*) with average removals that ranged from 1 to 2 logs. The two MGEs genes, *intI1* and *korB*, were also more resilient to the treatment, with removals in the range of 0.6-1.5 logs on average, except for WWTP1 in which *intI1* was significantly better removed (2.75 logs, $p > 0.01$). Therefore, although WWTP1 received a more considerable amount of *intI1* gene, it succeeded in removing it to the same or lower levels than the other two WWTPs.

For the greatest part of the measured genes, the wastewater treatment did not exacerbate but rather decreased the relative abundance of the studied ARGs. For some of the genes (*intI1*, *sul1,* or *sul2)*, a non-significant change or a slight relative increase was found in some of the WWTPs. In contrast, *korB* relative abundance increased significantly ($p < 0.001$) after the treatment in all 3 WWTPs (**supplementary information Figure S3.6**). These data confirm our previous observations for one-time measurements in 60 WWTPs for more extended sampling periods.

A sampling of intermediate steps within the treatment was performed to evaluate the contribution of each treatment step to the removal of both *E. coli* and genes. The primary treatment step (WWTP2) did not affect the removal of genes and exhibited a moderate but significant effect in removing the fecal indicator (-0.11 logs, $p<0.05$). The A-stage (AB line, WWTP3) moderately removed *E. coli* (-0.17 logs, $p <0.05$) and genes (-0.36 logs, $p <0.001$). Therefore, the greatest removal of both *E. coli* and the genes occurred in the biological nutrient removal stages. Activated sludge with short solid retention times and short clarification, as in the A-stage is thus not sufficient for the extensive removal of the pathogens or ARGs.

Lastly, we compared the removal efficiencies of two parallel lines- AB-line (based on flocculent sludge) and aerobic granular sludge- treating the same influent. Aerobic granular sludge is a modern water treatment technology requiring smaller space and footprint than conventional activated sludge systems (Pronk et al., 2015). Aerobic granular sludge is based on bacterial aggregation in granules instead of flocs. This configuration comprises a different spatial distribution and bacterial community that could affect the removal of ARGs compared to flocculent sludge. The presence of ARG in granules has so far only been studied concerning

accumulation during the granulation processes in bench-scale aerobic granular sludge reactors (Li et al., 2020). However, information on the occurrence of ARGs in the sludge fraction of full-scale AGS installations compared to conventional sludge is missing.

After a one-year of sampling, no significant differences were observed in removing ARGs and MGEs among the two parallel treatments. Exceptionally, *ermB* gene was better removed in the AB system than in the aerobic granular sludge system (+0.22 logs, $p<0.01$). The removal of *E. coli* was also similar to the AB system (Figure S5), in line with a recent study addressing the removal of fecal indicators (Barrios-Hernández et al., 2020).

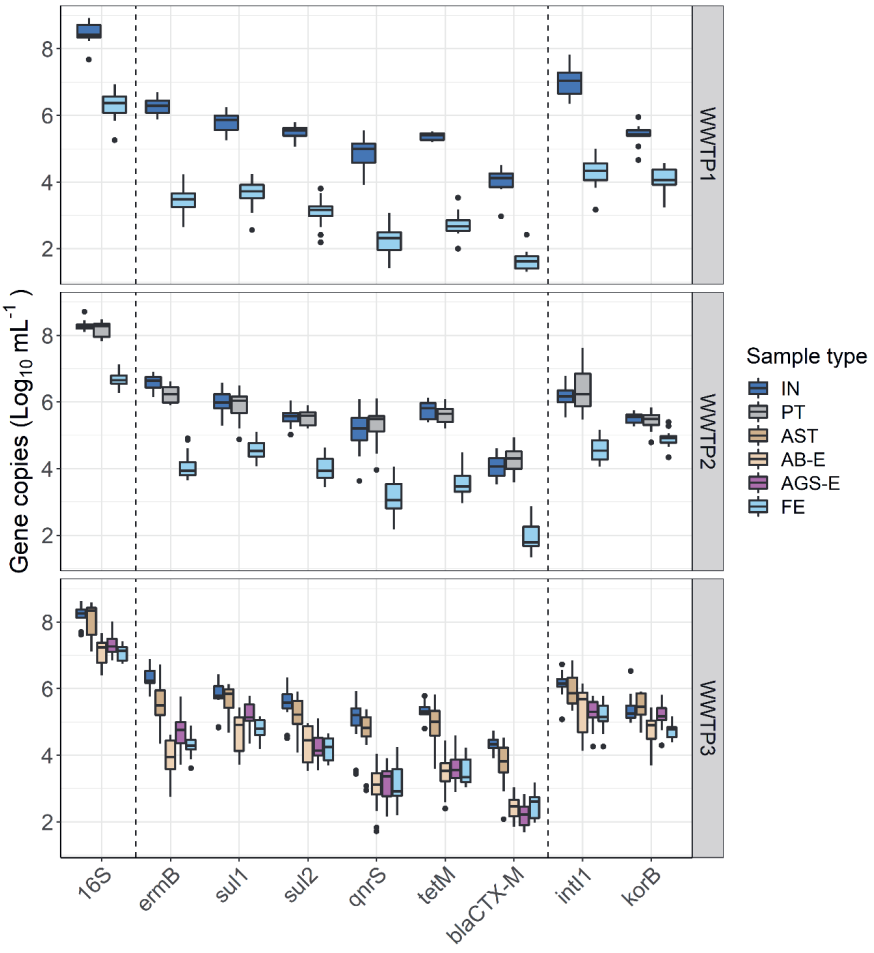


Figure 3.3. Absolute abundance of 16S rRNA, ARGs, and MGEs in the waterline of three Dutch WWTPs through a year.
Abbreviations: IN: Influent; PT: after Primary Treatment; AST: After A stage; AB-E: Effluent after AB treatment;

3.3.2.2. The removal of genes and E. coli *is compromised by high hydraulic loads and effluent suspended solids but not by seasonal temperature*

The effect of abiotic parameters (HLF, turbidity, and average temperature) on the removal of ARGs, MGEs, and *E. coli*, was investigated through linear mixed models (**supplementary information Table S3.3** models 8-10). Irrespective of the type of wastewater treatment, the removal of both *E. coli* and genes was hampered at high HLF (**Figure 3.4**). The removal capacity was modelled to decrease by 0.53 log CFUs ($p<0.001$) and 0.35 logs gene copies ($p<0.01$) at double the average daily flow. This gene removal rate is in good agreement with that obtained in our previous study (-0.38 logs) based on single measurements across many plants. Higher turbidity in the effluent was also correlated with a minor but significant decrease in the removal of *E. coli*, (-0.01 logs per $\Delta 1$ Nephelometric turbidity unit^{-1} $p<0.05$) and genes (-0.02 logs $p<0.05$), **Figure 3.4**. In contrast, seasonal changes in the average air temperature on the day of sampling did not alter the removal of *E. coli* nor genes (**supplementary information Figure S3.7**). Hence, opposite to what was observed for influent, variation in flow was the leading cause of variability, and the seasonal temperature had no contribution. The mechanisms by which the removal capacity of WWTPs might be disturbed with the increasing flow have been discussed previously (Pallares-Vega et al., 2019). In short, increasing flow causes wastewater to spend a shorter time in the biological treatment and sedimentation steps.

The lack of effect of seasonal temperature in the removal capacity might appear unexpected since fluctuations in seasonal temperature are known to significantly shape the bacterial community composition within the activated sludge (Griffin and Wells, 2017) and alter the treatment performance, *i.e.,* by nitrification failure during winter (Johnston et al., 2019). Surprisingly, the impact of seasonal temperature on ARGs and *E. coli* removal during full-scale wastewater treatment is seldom reported and therefore remains poorly understood. From the available studies, no statistical differences can be found regarding the seasonal occurrence of *E. coli* in full-scale WWTPs effluents (Lépesová et al., 2019; Osińska et al., 2020). Moreover, Barrios-Hernandez et al. (2020) described no effect of seasonal temperature on the removal of *E. coli*.

Seasonal peaks of absolute ARGs in effluent have been previously found in winter and spring (Harnisz et al., 2020) or summer (Jiao et al., 2018). In this study, seasonal fluctuations in absolute effluent concentrations (moderately higher with lower temperatures) were observed only in WWTP2 (**supplementary information Figure S3.7)**. Despite this trend, our results indicate that changes in the seasonal temperature did not influence the removal rates in any of the

WWTPs (**supplementary information Figure S3.7**). In contrast, Jiao et al. (2018) reported a better removal of ARGs during summer. However, in Jiao´s study, the effect of temperature cannot be detached from that of flow dynamics (highly significant according to our results), because information about the flow was not included. The degree in which temperature or flow influence the removal efficiency of the treatment might vary across countries with different temperature and precipitation regimes. Thus, additional studies in other locations accounting for both flow and temperature might be needed to understand further the role of temperature and flow in the removal of resistance determinants and *E. coli*.

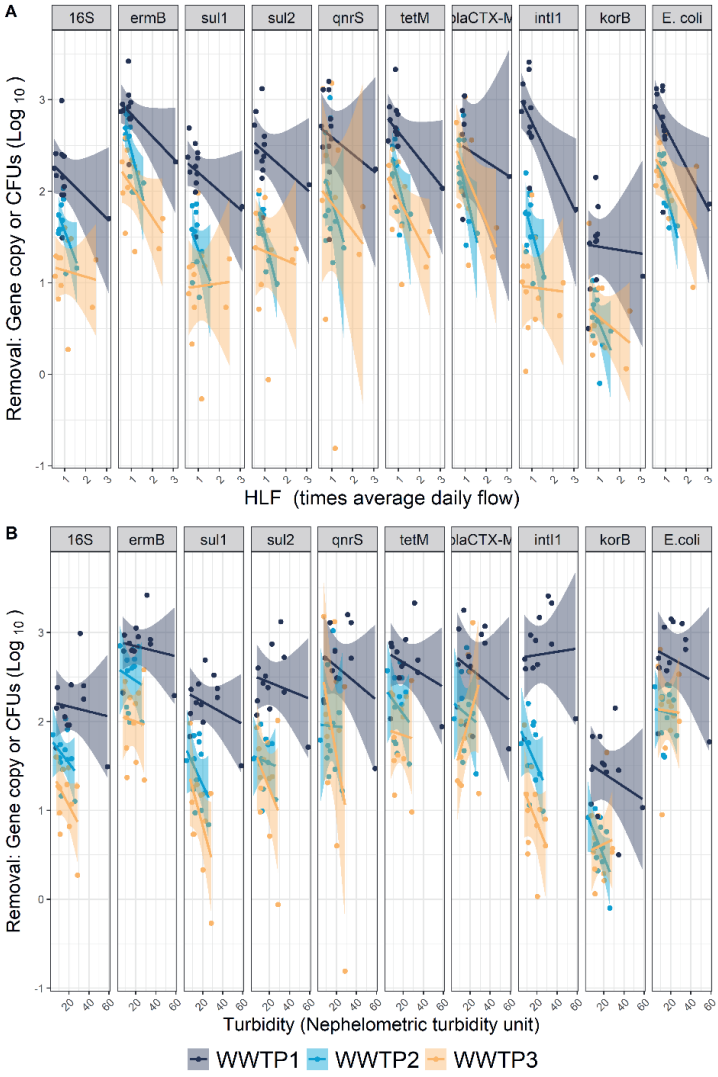


Figure 3.4. Removal efficiency of 16S rRNA, ARGs, MGEs and *E. coli* in three Dutch WWTPs in function of rainfall measured as hydraulic load factor (panel A) and effluent turbidity (panel B).

3.3.2.3. Fate of antimicrobials and disinfectants during wastewater treatment

The fate of the different antimicrobials and disinfectant residues during wastewater treatment depended on the compounds studied (**Figure 3.5 and Figure S3.8**). Some compounds were found both in effluent and biosolids (azithromycin, ciprofloxacin, sulphapyridine), while others were present either in the effluent (sulfamethoxazole, trimethoprim, clarithromycin) or in the biosolids line as the tetracyclines (tetracycline and doxycycline) and the disinfectants (BAC_{12} and BAC_{14}). Last, although erythromycin was not detected in any of the influent samples, it was sometimes present in AGS and DS from WWTP3. All types of treatments, including those based on granular sludge (**Figure S3.9**), reduced to a similar extent the antimicrobial concentrations (2-10-fold, depending on the compound). Specifics of the concentrations in each WWTP´s effluents are gathered in **supplementary information Table S3.1**. Despite the partial decrease, nine of the tested antimicrobials were still detectable in some of the effluent samples, although only ciprofloxacin and azithromycin were above the PNEC-MIC levels. Most of those compounds have not been commonly detected either in the upstream or downstream surface waters of the WWTPs discharge points (Sabri et al., 2018; Verburg et al., 2019). Hence, despite WWTPs discharging antimicrobials into the receiving waterbodies, the residues are diluted and/or sorbed to sediment, reducing their concentrations below the limits of detection.

The compounds that sorbed to the biosolids line were found in both DS and AS, although with higher concentrations in the DS samples than in the AS samples, likely derived from the difference in the solids content of each type of sample. Ciprofloxacin was again the most common antibiotic residue (2-4 mg kg^{-1}TS). Ofloxacin, while barely present in influent, was often detected in the biosolids line but in lower quantities than ciprofloxacin, in line with previous studies in Europe (Lindberg et al., 2005; Radjenović et al., 2009). In general, the concentration of tetracyclines and sulphapyridine followed the trends observed elsewhere (Göbel et al., 2005; Lindberg et al., 2005; Shafrir and Avisar, 2012). Tetracyclines were often found in values ranging between 0.1-1.2 mg kg^{-1}TS for both AS and DS samples, which is around 5 to 40 µg kg^{-1} of fresh digested sludge for tetracycline and doxycycline respectively. Concentrations of 15 µg L^{-1} of tetracycline (150 times below the minimum inhibitory concentrations) have proposed to enhance the growth of *tet* resistant bacteria (Gullberg et al., 2014) and to stimulate horizontal gene transfer events *in vitro* (Jutkina et al., 2016), although the bioavailability of these residues in the biosolids may reduce such an effect.

Besides the aforementioned antimicrobial residues, the two disinfectants tested were

also highly sorbed onto sludge. Concentrations ranged between 1-14 mg kg^{-1} TS in CAS-like AS samples and 3-23 mg kg^{-1} TS in DS samples. The highest levels were reported for the AS in the A stage of WWTP3 with up to 49 and 101 mg kg^{-1} TS for BAC_{12} and BAC_{14}, respectively (**supplementary information Figure S3.9 and Table S3.4**). These concentrations meet with literature reports (Martínez-Carballo et al., 2007) and reflect the important accumulation of these compounds in the sludge. A high occurrence of BACs has been shown to hamper methanogenesis in anaerobic digesters (Zhang et al., 2015). Moreover, field amendments of BACs rich biosolids could result in the accumulation of these compounds, especially in clay soils, which could potentially lead to the selection of *qac* genes and co-selection of ARGs (Mulder et al., 2018).

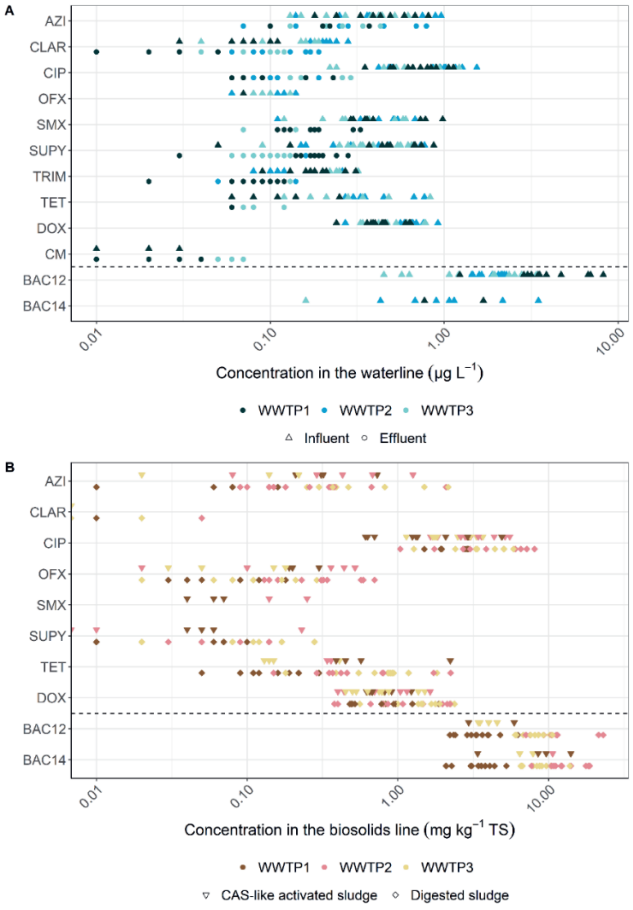


Figure 3.5. Antimicrobial and disinfectant residues in the waterline (panel A) and biosolids line (panel B) of three Dutch WWTPs. The detected compounds are presented in the y-axes, and their respective concentration in each type of sample is represented in the x-axes. Concentrations of antimicrobials are expressed in log_{10} scale. The CAS-like AS samples included in the panel (B) are the activated sludge samples from WWTP1, WWTP2, and the activated sludge from the B stage of WWTP3

3.3.2.4. The occurrence of resistance determinants in biosolids mirrored those in the influent

The occurrence of the ARGs in the biosolids line of the three WWTPs reflected the patterns observed in the influent (**supplementary information, Figure S3.10**). The highest concentrations were found for *ermB,* and *sul1* with 9.4-9.9 log copies g^{-1}TS, respectively (**Figure 3.5)**. On the lower rank were once again *qnrS* and bla_{CTXM}, which often laid below the limit of detection or quantification (up to 86% of some of the DS and AGS samples). Contrarily, a recent study analyzing a broad range of ARGs suggested no contribution of influent ARGs to the recycled activated sludge resistome, which was richer in abundance but poorer in diversity when compared to the influent (Quintela-Baluja et al., 2019). Since mixed liquors and not sedimented sludge were used in our study, a higher resemblance to influent ARGs patterns can be expected. Moreover, the reduced number of genes (8) included in this study in comparison with the comprehensive range (ca. 300) used by Quintela-Baluja and colleagues, might also have conditioned the observed resemblance. Our data also demonstrate a high occurrence of MGE elements in the biosolid line, particularly in the AS systems, where the *korB* gene was found in similar ranges than the integrase (*intI1)* gene. In contrast, *intI1* was 1-2 log more abundant than *korB* in influent samples. The high prevalence of *korB* in the activated sludge might explain the poor removal of this gene and the subsequent equalization of *intI1* and *korB* levels in the effluent. IncP-1 plasmids have been detected in biosolids of activated and digested sludge (Dröge et al., 2000). However, to the best of our knowledge, this is the first time IncP-1 plasmids have been quantified in activated sludge samples showing a high occurrence, which confirm their relevance in studies addressing horizontal gene transfer events in biosolids-like systems.

Neither the flow nor the temperature seemed to homogenously alter the concentration of genes in the CAS-like activated sludge systems (**supplementary information Table S3.3, Figure S3.11**); Despite some trends that could be observed across plants with increasing temperatures, the effect varied per studied gene (i.e. a decrease of *ermB* and *tetM* and an increase of *qnrS* absolute concentrations). Overall, there was a modest variation of concentrations of ARG in biosolids between WWTPs (roughly 0.5 logs) and across the year. However, the absolute abundance of most of the genes was greater in AS than DS. The same effect was observed for the *E. coli* concentrations (**supplementary information Figure S5, Table S5**). Lower absolute concentrations of ARGs, MGEs, and *E. coli* were also observed among AGS in contrast to the AS from the B stage (which is comparable to a conventional activated

sludge system). These differences are likely due to normalization per gram of TS, which is roughly 10-fold higher in the DS and AGS samples compared to AS ones. When normalized to the 16S rRNA (**supplementary information, Figure S3.12**), the relative concentration of several of the ARGs and MGEs in biosolids was similar among the aforementioned pairs (AS *vs.* DS and AGS *vs.* AS from the B stage). Only a slightly lower relative concentration of *ermB* was observed in AGS. A lower adhesion of bacteria harbouring *ermB* gene to the granular sludge fraction could explain its poorer removal after AGS treatment in comparison with the AB line.

Consistently higher relative concentrations of ARGs in AS A stage were observed in comparison to AS from B stage, and another flocculent AS from WWTP1 and WWTP2, and AGS (**Figure S3.12**). This difference could be due to the operational conditions of A stage, where the solids retention time is significantly shorter (0.3 days) than that used for B stage (23 days), and other conventional flocculent AS systems (15-20 days) or AGS (>30 days) (Barrios-Hernández et al., 2020; De Graaff et al., 2016). Shorter solids retention time most likely limits the natural decay of incoming antibiotic resistant bacteria by out competition of indigenous sludge microbiota and protozoa predation.

A persistent higher concentration of *tetM* after the anaerobic digestion was observed when compared with AS samples for both absolute and relative abundances **(Figure S3.12**). This suggests that the anaerobic treatment might select for bacteria harbouring this gene. As aforementioned, the concentrations of tetracycline residues in digested sludge might also contribute to the selection of *tet* genes, although similar effects were not found for the quinolone resistance. A slight enrichment of the relative abundance after anaerobic digestion was also observed for *ermB* (**Figure S3.9**). These findings are in accordance with the results of Ma et al. (2011) in bench-scale mesophilic digesters, where they even observed an increase in the absolute abundance of *erm* genes and some of the tested *tet* genes. In contrast, *intI1* and *sul* genes decreased in both relative and absolute abundance. An increase of several ARGs, including *erm*, *tet,* and *sul* genes was also observed in two full-scale anaerobic digestors in China (Tong et al., 2019), while in another full-scale study in the US, the relative abundance of three *tet* genes varied depending on the sampling dates (Ghosh et al., 2009). Digested sludge is used in some countries as crops fertilizer. The impact of pathogens and ARGs from sludge amendments in soil is still under debate (Rahube et al., 2014; Rutgersson et al., 2020). In The Netherlands, digested sludge undergoes incineration. However, there is a growing interest in nutrient recovery from this by-product; therefore, increasing the knowledge of possible hazards in the handling and downstream processing of digested sludge is important.

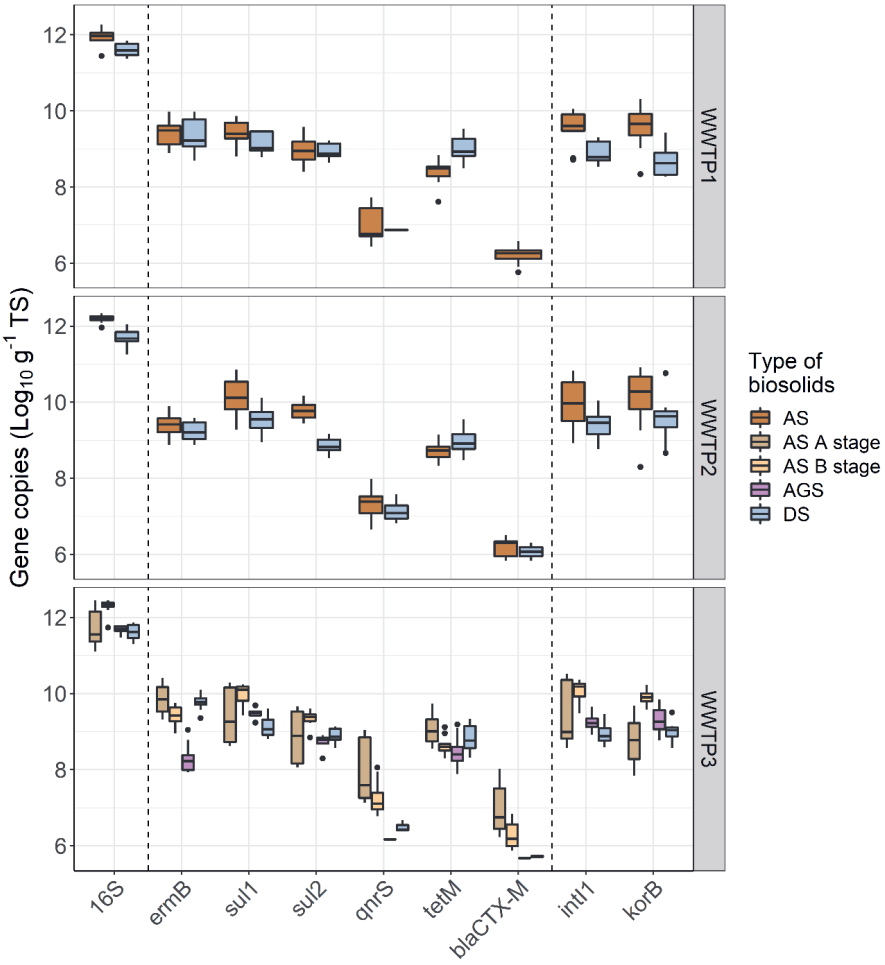


Figure 3.6. Absolute abundance of 16S rRNA, ARGs, and MGEs in biosolids line of three Dutch WWTPs through a year. Abbreviations: AS: Activated sludge; AS A stage: Activated sludge from the A stage of the AB system, AS B stage: Activated sludge from the B stage of the AB system; AGS: Aerobic Granular Sludge treatment; DS: Digested Sludge

3.3.3. Depicting sampling strategies

If the results obtained in this study are compared with those of our previous work (sampling multiple WWTPs but on a single occasion), the former managed to capture similar variability in ARGs occurrence and removal as in repeated sampling across one year (**supplementary information Figure S13**). Thus, shorter sampling efforts might be enough to evaluate the removal abilities of a WWTP. If the objective is to evaluate variability in performance (and address possible solutions), rainy and dry periods might be more interesting to assess than seasons.

In the absence of molecular methodology or the need for rapid results, the use of bacterial surrogates to evaluate the fate of ARGs might be necessary. Correlation analysis (Pearson's correlations) highlighted that *E. coli* should not be used to evaluate the variation in incoming concentrations of ARGs but could be considered as a surrogate to evaluate the removal of specific ARGs such *as bla*$_{CTX-M}$*, ermB, and tetM* (**supplementary information Figure S3.14-Figure S3.16**), commonly associated to Enterobacteriaceae and Lactobacillae. These taxa follow *E. coli* removal patterns during wastewater treatment (Barrios-Hernández et al., 2020; Ferreira Da Silva et al., 2007; Ottoson et al., 2006).

3.4. Conclusions

A one-year sampling campaign of three full-scale WWTPs highlighted that warmer seasonal temperature marginally decreased the concentrations of resistance genes in the influent but increased those of *E. coli.* However, seasonal temperature variation had an impaired effect on concentrations of antimicrobials in the influent. Instead, rainfall played a major role by diluting the concentrations of antimicrobials as well as fecal indicators such as *E. coli*, but not of resistance genes. Rainfall increasing the typical hydraulic load of each WWTPs significantly reduced the efficiency of wastewater treatment removal of genes and *E. coli,* in agreement with previous findings across The Netherlands. Increasing effluent´s turbidity was also related to slightly poorer removal. In addition, we concluded that the occurrence of resistant determinants in the biosolids line followed the occurrence patterns in the influent and that IncP-1 plasmids are highly abundant in biosolids. Finally, full-scale activated sludge and granular sludge technologies displayed comparable performance in the ability to remove antimicrobials, resistant determinants, and the fecal indicator *E. coli.*

3.5. Acknowledgements

We would like to thank the waterboards, Royal HaskoningDHV members, and especially to all the plant operators of the three WWTPs for their essential help during the sampling campaign and data gathering. We extend our gratitude to Stina Wegener for her tireless effort during the sample collection and to Goncalo Macedo for his assistance throughout the data analysis. We finally acknowledge Erwin Tuithof and the analytical team at Wetsus for the analysis of the antibiotic residues.

This work was co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslan, and the Northern Netherlands Provinces. Besides, this research has received funding from the European Union's Horizon2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 665874 and was co-funded by STOWA. The authors would also like to thank the members of the research theme Source Separated Sanitation for the shared knowledge and financial support.

3.6. Appendix A: DNA extraction quality control and qPCR reaction reagents and conditions.

Quality of the extractions

To assess the efficiency and quality of the DNA extraction, all the samples were spiked with an internal standard consisting of $1x10^7$ copies of synthetic gene fragments (gBlocks, IDT technologies, IA EE. UU) of the synthetic blue fluorescence protein (*bfp*) gene prior to extraction. DNA extracts were quantified using a Quantus™ fluorometer (Promega, NL) according to the supplier's instructions. DNA quality was assessed by gel electrophoresis (agarose at 1.5% m/v) and by measuring absorbance ratio at 260/280 nm and 260/230 nm using a Nanodrop spectrophotometer (ThermoScientific, UK)

qPCR: oligonucleotides, probes, and reaction and conditions

Preparation of qPCR reagent mix and reaction conditions was performed as indicated in Pallares-Vega et al.,2019, except for the *korB* assay, for which an increase of the primer concentration was used (400nm), and for the inclusion of the *bfp* assay, that follows the average reaction conditions with annealing temperature at 60ºC

Table A1: Oligonucleotides and probes used for gene detection by qPCR reactions. In primes/probes with degenerate code, Y stands for pyrimidine bases (C or T), R stands for purine (A or G), S for strong bases (C or G), and V for A, C, G (IUPAC nomenclature).

Target gene	Reference	Probe name	Oligonucleotide sequence 5'-3'	Conc. in reaction (nmol L^{-1})	Ann. T^a (in ºC)
16S rRNA	(Lane, 1991; Muyzer et al., 1993)	338F	ACTCCTACGGGAGGCAGCAG	300	60
		518R	ATTACCGCGGCTGCTGG		
qnrS	(Marti and Balcázar, 2013)	qnrSrtF11	GACGTGCTAACTTGCGTGAT	400	60
		qnrSrtR11	TGGCATTGTTGGAAACTTG		
tetM	(Peak et al., 2007)	tet(M)F	GGTTTCTCTTGGATACTTAAATCAATCR	500	60
		tet(M)R	CCAACCATAYAATCCTTGTTCRC		
sul1	(Pei et al., 2006)	Sul1-F	CGCACCGGAAACATCGCTGCAC	300	65
		Sul1-R	TGAAGTTCCGCCGCAAGGCTCG		
sul2	(Pei et al., 2006)	Sul2-F	TCCGGTGGAGGCCGGTATCTGG	400	61
		Sul2-R	CGGGAATGCCATCTGCCTTGAG		
ermB	(Knapp et al., 2010)	ErmB-F	AAAACTTACCCGCCATACCA	400	60
		ErmB-R	TTTGGCGTGTTTCATTGCTT		
bla $_{ctxM}$	(Marti and Balcázar, 2013)	q_CTXM-F	CTATGGCACCACCAACGATA	400	60
		q_CTXM-R	ACGGCTTTCTGCCTTAGGTT		
intI1	(Barraud et al., 2010)	IntI-F	GATCGGTCGAATGCGTGT	400	60
		IntI-R	GCCTTGATGTTACCCGAGAG		
korB (IncP-1)	(Jechalke et al., 2013)	IncP-F	TCATCGACAACGACTACAACG	400	55
		IncP-Fz	TCGTGGATAACGACTACAACG	400	
		IncP-R	TTCTTCTTGCCCTTCGCCAG	400	
		IncP-Rd	TTCTTG ACTCCCTTCGCCAG	400	
		IncP-Rge	TTYTTCYTGCCCTTGGCCAG	400	
		Probe-P	TCAGYTCRTTGCGYTGCAGGTTCTCVAT	400	
		Probe-Pgz	TSAGCTCGTTGCGTTGCAGGTTYUCAAT	400	
bfp	(De Rooij et al., 2019)	q_bfp	CAACGTCTATATCATGGCCGAC	300	60
		q_bfp	CAACGTCTATATCATGGCCGAC	300	

3.7. Appendix B: Sample preparation for antimicrobials and disinfectants measurements in biosolids

For the sludge line samples, aliquots stored at – 20ºC were defrosted overnight at 5ºC. AGS samples were homogenized by bead-beating at 4500 rpm for 30 s in the Precellys homogenizer (Bertin Technologies SAS, FR) with the help of 4mm glass beads (Merk, NL). AS and DS samples were homogenized by vigorous manual agitation. A total of 0.15 g of homogenized AGS and 0.75 g of homogenized AS or DS were used for the analysis.

The conditioning of the sludge matrix was achieved by mixing the sample with 1.5 mL of buffer (ammonium formate/formic acid (50:50, v/v), pH 2). To this mix, the following was added: 0.1 mL of an internal standard with isotopically labelled compounds (**Table B3**), 1.5 mL of modifier (consisting of in 100 mL: 50 mL of oxalic acid at 1 mol L^{-1}, 15 mL of ammonia at 5 mol L^{-1}, 5 mL of formic acid at 99% (v/v) and 35 mL of ultrapure deionized water), 3 mL of methanol at 99% v/v and 1 mL of organic modifier (acetonitrile/methanol (50:50 v/v) + 1 % formic acid). In order to calculate the recovery of each compound in each sample, a parallel vial was prepared, including 0.4 mL of a standard containing a mix of all the tested compounds. For both samples and recovery surrogates, the volume was completed up to 15 mL with ultrapure deionized water. The mix was vortexed for 30 min at speed 8 (1700 rpm) and subjected to sonication for 15 min in a bath sonicator (Bandelin electronic, DE). The vials were centrifuged at 3475 x g for 10 min, and the supernatants used directly for analysis by LC-MS/MS.

All samples were injected in an Agilent 6420 Triple Quadrupole LC-MS/MS system with an electrospray ion source. All the compounds were detected in the positive mode after separation in a ZORBAX Eclipse Plus C18 RRHD L = 50 x d = 2.1 mm column with 1.8 µm particle size. Detailed information about the mobile phases and data analyses can be found in **Table B1, Table B2, Table B3, Table B4.**

The limit of detection (LOD) and limit of quantification (LOQ) of the method were determined for each compound as the lowest detectable amount of compound with a signal-to-noise ratio of 3 and 10, respectively. The recovery rates for each compound in each sample were calculated from the spiked samples. The samples values were then recalculated by multiplying each result by the corresponding recovery, only if this was among 50-150%. When the recovery value was below or above the aforementioned threshold, the sample was excluded. For the disinfectant residues BAC_{12} and BAC_{14}, the recoveries were not applied, as the concentrations already present in the samples were 10-30 times higher than the spiked

concentrations. Thus, the results displayed are the concentration in the sample without further recalculations

Table B1: Composition of mobile phases and parameters for liquid chromatography (LC) separation of the antimicrobials and disinfectant residues.

Mobile phase	
Mobile Phase A	Positive electrospray ionization: 2,5 L ultrapure deionized water + 5mL formic acid (99% v/v), 0,5 mol L^{-1} ammonia 5mol L^{-1} + 0,1 mL Oxalic acid 1 mol L^{-1} Negative electrospray ionization: 2,5 L ultrapure deionized water + 5mL ammonia (5 mol L^{-1}) + 1 mL Formic acid (99% v/v) + 0,1 mL Oxalic acid 1 mol L^{-1}
Mobile Phase B	Positive electrospray ionization: Acetonitrile + 0,1% Formic acid Negative electrospray ionization: Acetonitrile

Table B2: Elution gradient program specifications in positive electrospray ionization for liquid chromatography (LC) separation of the antimicrobials and disinfectant residues.

Time (min)	Mobile phase B (%)	Pump (mL/min)	Pressure (bar)
0.10	5	0.250	500.00
1.0	65	0.250	500.00
8.0	75	0.250	500.00
8.5	5	0.250	500.00
13.0	5	0.250	500.00

Table B3: Isotopically labelled compounds used as internal standards.

Compound	Name	Concentration in Standard solution (µg mL^{-1})	Supplier
TRIM-D9	Trimethoprim-D9	0,253	Sigma-Aldrich
DIA	Diaveridine	0,253	Sigma-Aldrich
TRCD3	Triclosan-D3	0,253	Sigma-Aldrich
FNPF	Fenoprofen	1,262	Sigma-Aldrich
ATL-D7	Atenolol-D7	0,253	Sigma-Aldrich
CFX-D8	Ciprofloxacin-D8	0,253	Sigma-Aldrich
SUDOX-D3	Sulfadoxin-D3	0,253	Sigma-Aldrich

Table B4: Monitored ions and mass spectrometry parameters used for correction of peak areas of the antimicrobials and disinfectant residues.

Compound	Precursor Ion	Product Ion	Fragmentor voltage (V)	Collision energy (V)	Ret Time (min)	Polarity
AMOX	366.1	349.1	100	3	0.88	Positive
AMOX	366.1	208	100	8	0.88	Positive
AMP	350.1	160.1	100	10	2.95	Positive
AMP	350.1	106	100	22	2.95	Positive
ATd7	274	145	125	19	0.86	Positive
AZI	749.5	591.4	100	30	4.4	Positive
AZI	749.5	158.1	100	40	4.4	Positive
BaC12	304.3	212.2	140	15	6.03	Positive
BaC12	304.3	91	140	32	6.03	Positive
BaC14	332.3	240.2	140	18	6.55	Positive
BaC14	332.3	91	140	35	6.55	Positive
CFT	456.2	396.2	110	4	4.1	Positive
CFT	456.2	324.2	110	8	4.1	Positive
CIP	332.1	314.1	115	20	4.13	Positive
CIP	332.1	231	115	41	4.13	Positive
CIPd8	340	322	130	17	4.13	Positive
CLAR	748.5	158.1	150	28	4.88	Positive
CLAR	748.5	116.1	150	45	4.88	Positive
CM	425.3	377.2	110	20	4.48	Positive
CM	425.3	126.1	110	30	4.48	Positive
DIA	261.2	245.2	155	16	2.03	Positive
DM	141.9	96.2	100	15	1.62	Positive
DM	141.9	81.1	100	28	1.62	Positive
DOX	445.2	428	150	13	4.52	Positive
DOX	445.2	321.1	150	34	4.52	Positive
ERYT	734.5	576.4	165	15	4.68	Positive
ERYT	734.5	158.1	165	30	4.68	Positive
FLUMEQ	262.2	244	100	15	5.11	Positive
FLUMEQ	262.2	202	100	36	5.11	Positive
LINCOM	407.8	360.3	140	18	1.77	Positive
LINCOM	407.8	126.1	140	30	1.77	Positive
OFX	362.3	318.2	120	16	4.05	Positive
OFX	362.3	261.2	120	28	4.05	Positive
OTET	461.2	426.1	120	18	3.92	Positive
OTET	461.2	283.1	120	35	3.92	Positive
PENG	335.2	217	180	12	4.9	Positive
PENG	335.2	202	180	24	4.9	Positive
PENV	351.2	257	180	10	5.02	Positive
PENV	351.2	229	180	14	5.02	Positive
SMX	253.9	156.1	100	13	4.65	Positive
SMX	253.9	108.1	100	24	4.65	Positive
SUDOX	311.1	155.9	120	17	4.61	Positive
SUDOX	311.1	108	120	30	4.61	Positive
SUDOXd3	314.1	156	120	17	4.6	Positive
SULFAM	279	186	120	16	4.02	Positive
SULFAM	279	156	120	18	4.02	Positive

Compound	Precursor Ion	Product Ion	Fragmentor voltage (V)	Collision energy (V)	Ret Time (min)	Polarity
SUPY	250.1	184	90	14	2.25	Positive
SUPY	250.1	156	90	11	2.25	Positive
TET	445.2		130	26	4.2	Positive
TET	445.2	349.1	130	30	4.2	Positive
TILMIC	869.5	696.6	280	47	4.51	Positive
TILMIC	869.5	174.2	280	54	4.51	Positive
TRIM	291.1	275.1	140	24	2.98	Positive
TRIM	291.1	261.1	140	24	2.98	Positive
TRIMd9	300	264	145	26	2.74	Positive
TYLOS	916.5	772.3	240	34	4.72	Positive
TYLOS	916.5	173.6	240	36	4.72	Positive

3.8. Appendix C: Statistical analysis

The statistical analysis, linear models, and mixed models were conducted in R 3.6.5 (R Core Team, 2018) and Rstudio (http://www.rstudio.com) with the packages, stats, lmer, and lmerTest (Bates et al., 2016; Kuznetsova et al., 2017).

Only for the statistical analysis, the final effluent samples having $bla_{CTX\text{-}M}$ values below the LOQ were replaced by the LOQ value (6 out of 36 values). All other genes were above the LOQ in all samples before the removal was calculated.

For the comparison analysis of single-gene occurrences in influent, or the comparison of single genes and *E. coli* removal performance across all three WWTPs or across the two parallel lines of WWTP3 and is final effluent, an analysis of the variance was used (when normality was met) followed by Tukey post-hoc analysis. The comparisons were made using the log concentrations of genes mL^{-1} or the log-transformed removal values, respectively. If the distribution did not meet the normality, the group comparison was performed with a non-parametric test (Kruskal-Wallis).

The influence of abiotic factors in the incoming and removal of antimicrobials genes and *E. coli* as well as the role of intermediate steps in the removal of genes, were analyzed by linear models and linear mixed models. The summary of the models is displayed in **Table C1,** and the construction of the models is described below:

To evaluate the contribution of either the overall treatment or the intermediate steps (primary treatment in WWTP2 or A-stage in the AB line of WWTP3) to the removal of genes

determinants and *E. coli*, linear mixed models were used with observations clustered by sampling time-point. The log-transformed concentrations (log10 of gene copies of ARGs, MGEs, and log10 CFU counts of *E. coli* per mL) from each sample type were used as the response variable. The explanatory variables tested in the mixed model were “sample.type", fixed term, representing the location of the sample within the WWTP, and the "gene.type" (only in the gene models), “WWTP” and "sample.code.month" as independent random terms (random intercept modelled). The latter allowed the model to account for paired measurements from the same month of influent (IN) and final effluent (FE), primary treatment (PT) or A stage (AST). The result of these models are coefficients describing the gene reduction per location, and differences in gene concentrations per WWTP, across all resistance genes or *E. coli* and sampling time-point.

For single plants, similar linear mixed models (or linear models as for *E. coli*) were also used to investigate the influence of additional explanatory factors (sampling parameters) on the removal of either ARGs and MGEs or *E. coli* CFU counts. In this case, the response variable was the removal value, calculated as the log10 of the ratio of the concentration of genes or CFU counts in the influent versus the final effluent. The explanatory variables (fixed terms) were the WWTP, the average temperature, the turbidity (as a surrogate for TSS presence in effluent), and the hydraulic load factor (HLF). The random terms were the “gene type” (only in the gene model) and the “sample.code.month” that allowed grouping all the genes from the same WWTP and sampling time point. For *E. coli*, only one explained variable (CFU counts) was available, and thus neither “gene type” nor “sample.code.month” random terms were applicable. The inclusion of WWTP as fixed term led to singular fit problem. Thus, for *E. coli*, a linear model was used instead.

The role of explanatory factors for the concentrations of resistance genes and *E. coli* in influent was also investigated through linear mixed models. The response variable was the log10-transformed influent concentration per mL of either ARGs and MGEs gene copies or CFU counts. In addition, the “load.pe” or absolute daily amount of resistance genes and CFU per population equivalent was used as a response variable. The load was obtained from multiplying influent concentrations with the flow on the measurement day, thereby correcting for increased treatment volumes **(Eq 3.1).** Last, the load was normalized per population equivalent (pe) “load.pe”, (where 1 pe stands for 150g of total oxygen demand), and used as a response variable (log10 transformed) **(Eq 3.2)**. The average atmospheric temperature and HLF from the day of sampling were used as the fixed term explanatory variables. Again, “gene type” (only in

the gene models) and "sample.code.month" were used as the random terms whilst HLF and temperature were used as fixed effects.

Eq (3.1): $Gene.load = log10(gene\ copies\ mL^{-1} * \frac{1000mL}{L} * \frac{1000L}{m^3} * \left(\frac{daily.flow\ m^3}{day}\right))$

Eq (3.2): $Gene.load.pe = log10\ (\frac{10^{\frac{Gene.load}{pe\ WWTP}}}{})$

The same explanatory factors were also modelled against the concentration of genes in sludge, by using a similar approach as for the influent models, but with the log10 transformed concentration of genes and CFUs per g of TS as a response variable. Due to high percentage of missing values, *qnrS* and *bla*$_{CTX-M}$ genes were not included in the model.

Finally, a linear mixed model to address the effect of both atmospheric temperature and HLF in the incoming concentrations of antimicrobials commonly found in influent (Azithromycin, Clarithromycin, Ciprofloxacin, Sulfamethoxazole, Sulpyridine, Trimethoprim, Doxycycline, Tetracycline, and Clindamycin). Missing values were replaced by LOD/√2 of each compound. The response variable was the concentration of antimicrobial in μg $L^{-1,}$ and the explanatory variables were the HLF and atmospheric temperature in the fixed terms, and "antimicrobial.type" to group for each type of antibiotic and "sample.code.month" in the random terms to account for samples from the same location and sampling time-point. After analysis, the model was considered to not being well-fitted, according to the optical inspection of residuals, and thus, results of this model are not further included in the results and discussion section.

In the linear mixed models with several explanatory variables, the relevance of the explanatory variables was determined through stepwise backward model reduction, and the quality of the models was assessed by visual inspection of the normality of the residuals.

The datasets used in this study are available in Mendeley Data repository doi:10.17632/53fk4cht32.1

Table C1: Linear models and linear mixed models used in this study. Abbreviations: Model Nb: model number; FM: full model; RM: Reduced model

Variable	Model Nb	Model type	Model formula
Influent- factors	1	FM	log gene copies mL^{-1} ~ HLF + T.Aver + WWTP + (1|gene.type) + (1|sample.code.month)
	2	RM	log gene copies mL^{-1} ~ T.Aver + (1|gene.type) + (1|sample.code.month)
	3	FM	gene load.pe ~ HLF + T.Aver + WWTP +(1| gene.type) + (1|sample.code.month)
	4	RM	gene load.pe ~ HLF + T.Aver + (1| gene.type) + (1|sample.code.month)
	5	FM/RM	log.CFUs mL^{-1} ~ HLF + T.Aver + WWTP
	6	FM	CFUs load.pe~ HLF + T.Aver + WWTP
	7	RM	CFUs load.pe~ HLF + T.Aver
Removal-factors	8	FM	removal log gene copies ~ HLF + T.Aver + Turbidity + WWTP + (1| gene.type)
	9	RM	removal log gene copies ~ HLF + Turbidity + WWTP + (1| gene.type)
	10	FM/RM	removal log CFUs ~ HLF + T.Aver + Turbidity + WWTP
Removal- steps	11	FM	log gene copies mL^{-1} ~ sample.type* + (1| gene.type)) + (1|sample.code.month)
	12	FM	log CFUs mL^{-1} ~ Sample.type* + WWTP+ (1|sample.code.month) *sample.type: IN vs FE
	13	FM	log gene copies mL^{-1}~ sample.type** + WWTP + (1| gene.type) + (1|sample.code.month)
	14	FM	(log CFUs mL^{-1} ~ sample.type** + (1|sample.code.month) **sample.type: IN vs PT
	15	FM	log gene copies mL^{-1} ~ sample.type*** + (1| gene.type)) + (1| sample.code)
	16	FM	log CFUs mL^{-1} ~ sample.type*** + (1|sample.code) ***sample.type: IN vs AST
Activated sludge - factors	17	FM	log gene copies gTS^{-1} ~ HLF + T.Aver + WWTP + (1|gene.type) + (1|sample.code.month)
	18	FM	log gene copies gTS^{-1} ~ HLF + T.Aver + WWTP
Antimicrobials	19	FM	Antimicrobial concentration µg L^{-1} ~ HLF + T.Aver + WWTP + (1|antimicrobial.type) + (1|sample.code.month)

3.9. Supplementary information

Table S3.1. Occurrence and concentration of antimicrobials and disinfectants in the waterline of the three WWTPs through a year. Acronyms are n: the number of samples; >LOD: number of samples above the limit of detection. AZI: Azithromycin, CLAR: Clarithromycin, CIP: Ciprofloxacin: SMX: Sulfamethoxazole; SULPY: Sulphapyridine; TRIM: Trimethoprim: DOX: Doxycycline; TET: Tetracycline; CM: Clindamycin: BAC_{12}: Benzalkonium chloride 12; BAC_{14}: Benzalkonium chloride 14. PNEC-MIC concentrations based on (Bengtsson-Palme et al., 2016). Average concentrations only calculated when n≥2.

	PNEC-MIC µg L^{-1}	AZI 0.25	CLAR 0.25	CIP 0.06	OFX 0.50	SMX 16.00	SULPY NA	TRIM 0.50	DOX 2.00	TET 1.00	CM 1.00	BAC_{12} NA	BAC_{14} NA
WWTP1													
	Influent												
	n	12	12	12	12	12	12	12	12	12	12	12	12
	Recovery 50-150%	11	11	12	12	10	10	10	11	6	10	11	3
	>LOD	10	10	11	2	8	10	10	11	6	10	11	3
	Average (µg L^{-1})	0.46	0.08	0.73	0.20	0.52	0.49	0.19	0.45	0.13	0.02	4.34	1.07
	Effluent												
	n	12	12	12	12	12	12	12	12	12	12	12	12
	Recovery 50-150%	12	12	12	12	12	12	12	12	12	12	9	6
	>LOD	4	6	8	0	9	12	10	0	1	8	0	0
	Average (µg L^{-1})	0.22	0.02	0.12	NA	0.19	0.17	0.09	NA	NA	0.03	NA	NA
WWTP2													
	Influent												
	n	12	12	12	12	12	12	12	12	12	12	12	12
	Recovery 50-150%	10	11	12	12	12	12	12	12	11	12	12	10
	>LOD	9	11	12	9	11	12	11	12	9	3	12	7
	Average (µg L^{-1})	0.50	0.19	0.92	0.11	0.37	0.43	0.17	0.64	0.42	0.02	1.99	1.41
	Prim treat												
	n	12	12	12	12	12	12	12	12	12	12	12	12
	Recovery 50-150%	8	11	12	12	12	12	12	12	12	12	12	9
	>LOD	8	11	12	7	11	12	10	12	9	7	12	3
	Average (µg L^{-1})	0.64	0.17	0.64	0.10	0.35	0.45	0.17	0.54	0.25	0.01	1.60	1.51

	PNEC-MIC µg L^{-1}	AZI 0.25	CLAR 0.25	CIP 0.06	OFX 0.50	SMX 16.00	SULPY NA	TRIM 0.50	DOX 2.00	TET 1.00	CM 1.00	BAC_{12} NA	BAC_{14} NA
	Effluent												
	n	11	11	11	11	11	11	11	11	11	11	11	11
	Recovery 50-150%	11	11	11	11	11	11	11	11	11	11	11	11
	>LOD	9	11	9	0	3	11	3	0	0	10	0	0
	Average (µg L^{-1})	0.36	0.11	0.12	NA	0.12	0.15	0.10	NA	NA	0.03	NA	NA
WWTP3													
	Influent												
	n	12	12	12	12	12	12	12	12	12	12	12	12
	Recovery 50-150%	11	12	12	12	12	12	12	12	11	12	12	8
	>LOD	9	12	12	4	11	12	10	12	8	9	12	2
	Average (µg L^{-1})	0.35	0.11	0.71	0.10	0.38	0.37	0.21	0.49	0.32	0.02	1.82	0.16
	A stage												
	n	12	12	12	12	12	12	12	12	12	12	12	12
	Recovery 50-150%	11	12	12	12	12	12	12	12	10	12	12	2
	>LOD	7	12	10	0	9	12	9	12	7	7	2	0
	Average (µg L^{-1})	0.43	0.09	0.39	NA	0.30	0.26	0.15	0.49	0.18	0.02	1.12	NA
	AB effluent												
	n	12	12	12	12	12	12	12	12	12	12	12	12
	Recovery 50-150%	11	12	12	12	12	11	12	12	12	12	7	6
	>LOD	6	12	8	0	5	11	6	0	3	9	0	0
	Average (µg L^{-1})	0.27	0.06	0.29	NA	0.13	0.15	0.09	NA	0.10	0.04	NA	NA
	AGS effluent												
	n	12	12	12	12	12	12	12	12	12	12	12	12
	Recovery 50-150%	12	12	12	12	11	11	12	12	11	12	10	5
	>LOD	6	12	10	0	1	9	7	1	2	10	0	0
	Average (µg L^{-1})	0.30	0.06	0.17	NA	NA	0.11	0.09	NA	0.10	0.04	NA	NA
	Total Effluent												
	n	12	12	12	12	12	12	12	12	12	12	12	12
	Recovery 50-150%	11	12	12	12	12	12	12	12	11	12	8	6

	PNEC-MIC µg L^{-1}	AZI 0.25	CLAR 0.25	CIP 0.06	OFX 0.50	SMX 16.00	SULPY NA	TRIM 0.50	DOX 2.00	TET 1.00	CM 1.00	BAC_{12} NA	BAC_{14} NA
	>LOD	5	11	7	0	4	11	5	0	3	10	0	0
	Average (µg L^{-1})	0.28	0.06	0.15	NA	0.10	0.11	0.10	NA	0.09	0.04	NA	

Table S3.2. Average annual concentrations of 16S rRNA, ARGs, and MGEs in the waterline samples. Concentrations are expressed in gene log10 gene copy mL^{-1}. Abbreviations of the sample type: IN: Influent; PT: After primary treatment; AST: After A stage; AB-E: Effluent after AB treatment; AGS-E: Effluent after Aerobic Granular Sludge treatment; FE: Final Effluent; Other abbreviations: SD: Standard deviation

	WWTP1				WWTP2						WWTP3									
	IN		FE		IN		PT		FE		IN		AST		AB-E		AGS-E		FE	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
16S	8,49	0,33	6,33	0,45	8,31	0,16	8,22	0,24	6,66	0,31	8,22	0,29	8,04	0,50	7,12	0,40	7,36	0,30	7,09	0,24
ermB	6,26	0,25	3,42	0,41	6,58	0,23	6,24	0,25	4,09	0,43	6,32	0,30	5,55	0,67	3,90	0,61	4,70	0,52	4,31	0.36
sul1	5,80	0,28	3,59	0,43	5,98	0,33	5,88	0,46	4,56	0,34	5,75	0,46	5,61	0,53	4,69	0,56	5,18	0,40	4,79	0,28
sul2	5,51	0,19	3,09	0,45	5,55	0,27	5,54	0,23	3,99	0,37	5,51	0,50	5,16	0,59	4,34	0,52	4,21	0,43	4,18	0,35
qnrS	4,86	0,47	2,30	0,46	5,13	0,64	5,28	0,58	3,18	0,56	4,98	0,73	4,60	0,76	3,04	0,71	3,19	0,51	3,15	0,59
tetM	5,37	0,12	2,72	0,37	5,75	0,25	5,63	0,27	3,59	0,47	5,35	0,25	4,90	0,62	3,42	0,55	3,65	0,46	3,49	0,39
bla$_{CTXM}$	4,03	0,39	1,67	0,36	4,06	0,33	4,26	0,39	1,97	0,44	4,32	0,25	3,69	0,68	2,18	0,52	2,06	0,48	2,22	0,56
intI1	7,03	0,43	4,28	0,47	6,17	0,33	6,40	0,63	4,56	0,36	6,13	0,39	5,96	0,45	5,33	0,70	5,25	0,42	5,18	0,43
korB-IncP1	5,44	0,29	4,03	0,41	5,47	0,22	5,39	0,29	4,87	0,28	5.39	0,42	5,28	0,47	4,59	0,72	5,19	0,38	4,79	0,27

Table S3.3. Linear models and linear mixed model outcomes. Acronyms: beta: model estimate. SE: standard error of the estimate. z: z statistics. p: p-value. CI: Confidence Interval. HLF: hydraulic load factor. T.Aver: average air temperature; Load.pe: gene loads per population equivalent. Sample.code.month stands for the monthly sampling in each WWTP (i.e. results from WWTP1 month 1 have the same code). Sample.type: refers to the type of sample such as primary treatment (PT), Final effluent (FE) or effluent from A stage (AST), compared to the reference value of influent (IN) values.

Variable	nb	Model formula	Factor	Beta	SE	Z	P value	CI upper	CI lower
Influent-factors	1	log gene copies mL-1 ~ HLF + T.Aver + WWTP + (1|gene.type) + (1|sample.code.month)	HLF	0.107	0.079	2.406	0.186	0.053	0.268
			T.Aver	-0.017	0.007	- 4.128	0.025	-0.031	-0.002
		(full model)	WWTP2	0.076	0.104	1.298	0.472	-0.134	0.285
			WWTP3	-0.078	0.104	-1.340	0.458	-0.289	0.132
	2	log gene copies mL-1 ~ T.Aver + (1|gene.type) + (1|sample.code.month) (reduced model)	T.Aver	-0.016	0.007	2.094	0.044	-0.031	0.001
	3	gene load.pe ~ HLF + T.Aver + WWTP +(1| gene.type) + (1|sample.code.month)	HLF	0.418	0.082	5.070	<0.001	0.252	0.584
			T.Aver	-0.016	0.007	-2.165	0.037	-0.031	-0.001
		(full model)	WWTP2	0.024	0.108	0.227	0.821	-0.193	-0.242
			WWTP3	0.016	0.108	0.154	0.879	0.201	0.234
	4	gene load.pe ~ HLF + T.Aver + (1| gene.type) + (1|sample.code.month)	HLF	0.418	0.082	5.123	<0.001	0.254	0.582
		(reduced model)	T.Aver	-0.016	0.007	-2.157	0.038	-0.031	-0.001
	5	Log CFUs mL-1 ~ HLF + T.Aver + WWTP	HLF	-0.253	0.034	-7.31	<0.001	-0.323	-0.183
			T.Aver	0.027	0.003	8.293	<0.001	0.021	0.034
		(full and reduced model)	WWTP2	0.141	0.045	3.133	<0.001	0.049	0.232
			WWTP3	-0.043	0.04	-0.966	0.342	-0.013	0.005
	6	CFUs load.pe~ HLF + T.Aver + WWTP	HLF	0.057	0.039	1.460	0.154	-0.023	0.138

Variable	nb	Model formula	Factor	Beta	SE	Z	P value	CI upper	CI lower
		(full model)	T.Aver	0.029	0.004	7.959	<0.001	0.021	0.004
			WWTP2	0.089	0.051	1.730	0.093	0.016	0.194
			WWTP3	0.051	0.051	0.995	0.327	-0.054	0.157
	7	CFUs load.pe~ HLF + T.Aver	HLF	0.055	0.04	1.383	0.176	-0.026	0.135
		(reduced model)	T.Aver	0.029	0.004	7.942	<0.001	0.021	0.036
Removal - factors	8	removal log gene copies ~ HLF + T.Aver + Turbidity + WWTP + (1| gene.type) + (1|sample.code.month)	HLF	-0.353	0.102	-3.463	0.001	-0.457	-0.249
			T.Aver	-0.013	0.009	-1.379	0.176	-0.022	-0.003
		(full model)	Turbidity	-0.016	0.003	-2.842	0.007	-0.023	-0.010
			WWTP2	-0.835	0.070	-6.199	<0.001	-0.097	-0.697
			WWTP3	-1.040	0.070	-7.972	<0.001	-1.179	-0.912
	9	removal log gene copies ~ HLF + Turbidity + WWTP + (1| gene.type) + (1|sample.code.month)	HLF	-0.350	0.104	-3.346	0.002	-0.457	-0.249
			Turbidity	-0.015	0.006	-2.482	0.018	-0.023	-0.010
		(reduced model)	WWTP2	-0.835	0.138	-5.980	<0.001	-0.097	-0.697
			WWTP3	-1.04	0.134	-7.689	<0.001	-1.179	-0.912
	10	removal log CFUs ~ HLF + T.Aver + Turbidity + WWTP	HLF	-0.531	0.106	-4.990	<0.001	-0.748	-0.314
			T.Aver	-0.002	0.009	0.221	0.826	-0.018	0.021
		(full and reduced model)	Turbidity	-0.013	0.006	-2.245	0.032	-0.026	-0.001
			WWTP2	-0.733	0.14	-5.213	<0.001	-1.020	-0.446
			WWTP3	-0.589	0.13	-4.308	<0.001	-0.868	-0.868
Removal-steps	11	log gene copies mL-1 ~ sample.type + (1| gene.type) + (1|sample.code.month)	FE	-1.864	0.04	-44.60	<0.001	-1.940	-1.780
			WWTP2	0.400	0.051	7.816	<0.001	0.299	0.500

Variable	nb	Model formula	Factor	Beta	SE	Z	P value	CI upper	CI lower
			WWTP3	0.425	0.051	8.310	<0.001	0.325	0.525
	12	(log CFUs mL-1 ~ sample.type + WWTP+ (1|sample.code.month)	FE	-2.31	0.087	-26.324	<0.001	-2.486	-2.145
			WWTP2	0.447	0.011	4.157	<0.001	0.238	0.657
			WWTP3	0.223	0.011	2.065	0.044	0.013	0.431
	13	log gene copies mL-1~ sample.type + (1| gene.type) + (1|sample.code.month)	PT	-0.005	0.046	-0.11	0.912	-0.096	0.085
	14	(log CFUs mL-1 ~ sample.type + (1|sample.code.month)	PT	-0.105	0.004	-2.588	0.025	-0.189	-0.022
	15	log gene copies mL-1 ~ sample.type + (1| gene.type) + (1| sample.code)	AST	-0.357	0.063	-5.721	<0.001	-0.480	-0.234
	16	(log CFUs mL-1 ~ sample.type + (1|sample.code)	AST	-0.165	0.056	-2.908	0.015	-0.280	-0.048
Activated sludge - factors	17	log gene copies gTS -1 ~ HLF + T.Aver + WWTP + (1|gene.type) + (1|sample.code.month)	HLF	-0.011	0.074	-0.154	0.879	-0.160	0.137
			T.Aver	-0.004	0.007	-0.618	0.540	-0.019	0.010
			WWTP2	0.464	0.107	4.307	<0.001	0.247	0.681
			WWP3	0.336	0.107	3.117	0.003	0.118	0.553
	18	log gene copies gTS -1 ~ HLF + T.Aver + WWTP	HLF	-0.003	0.090	-0.036	0.972	-0.188	0.181
			T.Aver	0.003	0.008	0.323	0.749	0.003	0.009
			WWTP2	0.428	0.132	3.249	0.003	0.429	0.132
			WWP3	0.410	0.132	3.110	0.004	0.410	0.132

Table S3.4. Occurrence and concentration of antimicrobials and disinfectants in the biosolids line of the three WWTPs through a year. Acronyms are: n: number of samples; >LOD: number of samples above the Limit of Detection. AZI: Azithromycin, CLAR: Clarithromycin, ERY: Erythromycin; CIP: Ciprofloxacin; OFX: Ofloxacin; SMX: Sulfamethoxazole; SULPY: Sulphapyridine; TRIM: Trimethoprim: DOX: Doxycycline; TET: Tetracycline; OTR: Oxytetracycline; CM: Clindamycin: BAC_{12}: Benzalkonium chloride 12; BAC_{14}: Benzalkonium chloride 14. Average concentrations only calculated when n≥2.

		AZI	CLAR	ERY	CIP	OFX	SMX	SULPY	DOX	TET	OTR	CM	BAC12	BAC14
WWTP1														
	AS													
	n	12	12	12	12	12	12	12	12	12	12	12	12	12
	Recov. 50-150%	12	12	11	12	11	12	12	12	12	12	12	NA	NA
	>LOD	3	0	0	11	4	3	2	9	4	0	0	2	4
	Average (mg kg-1TS)	0.42	NA	NA	2.15	0.22	0.09	0.05	0.79	0.91	NA	NA	4.44	8.91
	DS													
	n	12	12	12	12	12	12	12	12	12	12	12	12	12
	Recov. 50-150%	12	12	11	10	12	11	12	12	11	12	12	NA	NA
	>LOD	6	1	1	10	12	0	12	12	11	2	7	12	12
	Average (mg kg-1 TS)	0.07	NA	NA	2.55	0.09	NA	0.06	0.80	0.13	0.03	0.01	3.52	3.43
WWTP2														
	AS													
	n	12	12	12	12	12	12	12	12	12	12	12	12	12
	Recov. 50-150%	12	12	10/1	12	12	11	12	12	12	12	12	NA	NA
	>LOD	6	1	1	12	6	3	3	6	2	0	0	0	1
	Average (mg kg-1TS)	0.55	NA	NA	3.32	0.22	0.17	0.88	0.89	0.35	NA	NA	NA	NA
	DS													
	n	12	12	12	12	12	12	12	12	12	12	12	12	12
	Recov. 50-150%	12	12	12/1	9	12	12	12	12	12	12	12	NA	NA
	>LOD	12	4	1	12	12	0	10	12	12	2	4	12	12
	Average (mg kg-1 TS)	0.43	0.02	NA	4.47	0.31	NA	0.07	1.19	0.76	0.02	0.01	13.47	13.67
WWTP3														
	AS A stage													
	n	12	12	12	12	12	12	12	12	12	12	12	12	12
	Recov. 50-150%	12	11	12	10	12	12	12	12	12	12	12	NA	NA
	>LOD	0	1	2	12	4	2	3	10	4	0	3	12	11

	AZI	CLAR	ERY	CIP	OFX	SMX	SULPY	DOX	TET	OTR	CM	BAC12	BAC14
Average (mg kg-1TS)	NA	NA	0.47	1.18	0.12	0.24	0.08	0.58	0.21	NA	0.03	29.31	51.67
AS B stage													
n	12	12	12	12	12	12	12	12	12	12	12	12	12
Recov. 50-150%	12	12	11	10	11	12	12	10	12	10	12	NA	NA
>LOD	3	1	0	9	5	0	2	10	4	0	4	4	2
Average (mg kg-1TS)	0.13	NA	NA	2.33	0.09	NA	0.04	0.80	0.21	NA	0.03	3.88	7.17
AGS													
n	10	10	10	10	10	10	10	10	10	10	10	10	10
Recov. 50-150%	10	10	7	10	10	10	10	10	10	10	10	NA	NA
>LOD	4	0	7	9	3	0	1	10	9	0	0	10	10
Average (mg kg-1TS)	0.04	NA	0.12	3.35	0.14	NA	NA	0.50	0.18	NA	NA	2.61	6.40
DS													
n	12	12	12	12	12	12	12	12	12	12	12	12	12
Recov. 50-150%	11	12	12	10	12	9	12	12	12	12	12	NA	NA
>LOD	9	4	8	9	11	0	11	11	6	5	7	12	12
Average (mg kg-1 TS)	0.67	0.01	0.14	3.75	0.14	NA	0.10	1.31	0.14	0.19	0.01	8.83	8.40

Table S3.5. Average annual concentrations of 16S rRNA, ARGs, and MGEs in the waterline samples. Concentrations are expressed in gene log10 gene copy g^{-1} TS. Abbreviations of the sample type: AS: Activated Sludge; DS: Digested Sludge: ASAST: Activated sludge AS; ASBST: Activated sludge B stage; AGS: Aerobic granular sludge. SD: Standard deviation.

	WWTP1				**WWTP2**				**WWTP3**							
	AS		DS		AS		DS		ASAST		ASBST		AGS		DS	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
16S	11.96	0.20	11.60	0.17	12.19	0.10	11.69	0.20	11.70	0.46	12.29	0.18	11.57	0.19	11.60	0.18
ermB	9.39	0.32	9.36	0.41	9.38	0.29	9.23	0.23	9.85	0.36	9.43	0.24	8.29	0.35	9.77	0.18
sul1	9.40	0.34	9.14	0.25	10.16	0.44	9.52	0.33	9.38	0.65	9.98	0.26	9.57	0.23	9.14	0.27
sul2	8.98	0.34	8.94	0.20	9.77	0.22	8.85	0.21	8.86	0.64	9.35	0.18	8.74	0.16	8.90	0.18
qnrS	7.03	0.46	NA	NA	7.31	0.37	7.14	0.28	7.92	0.77	7.22	0.40	NA	NA	6.50	0.13
tetM	8.40	0.31	9.01	0.32	8.72	0.24	8.96	0.30	9.06	0.40	8.61	0.23	8.48	0.38	8.81	0.33
bla_{CTXM}	6.21	0.25	NA	NA	6.17	0.24	6.07	0,.24	6.97	0.63	6.28	0.23	NA	NA	5.72	0.03
intI1	9.56	0.41	8.87	0.26	9.98	0.59	9.40	0.38	9.42	0.75	10.07	0.25	9.25	0.20	8.95	0.29
korB-incP1	9.60	0.53	8.67	0.36	10.09	0.73	9.56	0.52	8.78	0.60	9.90	0.18	9.29	0.32	8.99	0.23

Figure S3.1. Map with the location of the Dutch wastewater treatment plants (WWTPs) sampled in this study.

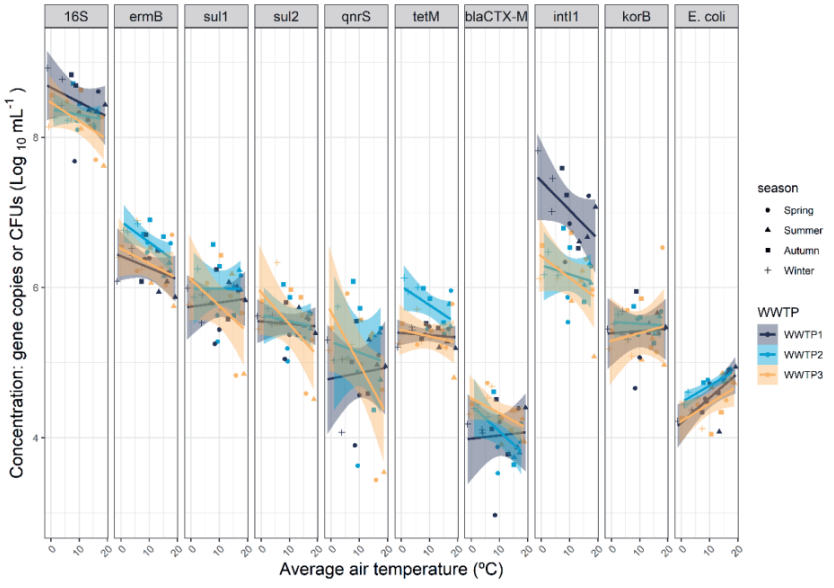


Figure S3.2. influent concentrations of 16S rRNA, ARGs, MGEs, and the fecal indicator *E. coli* in function of the air temperature.

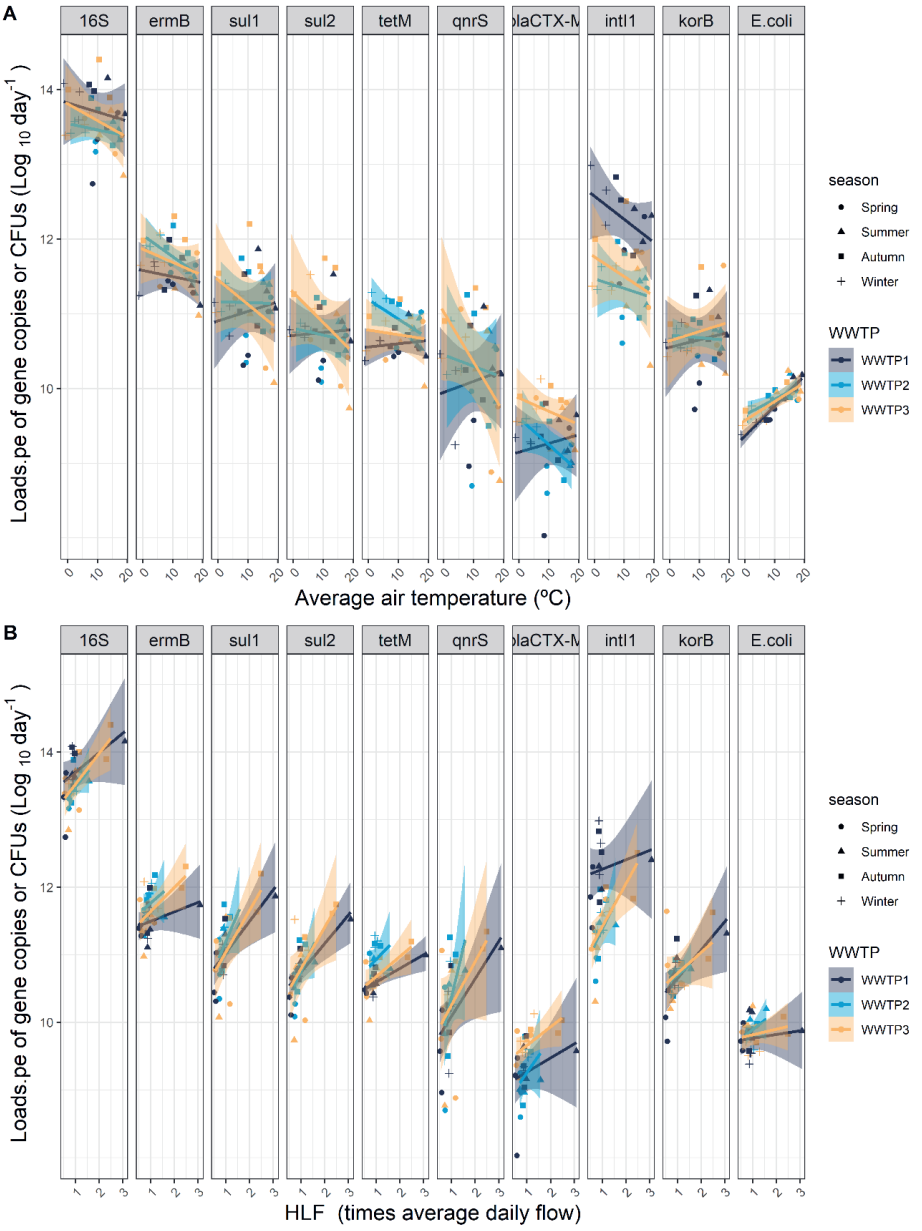


Figure S3.3. Influent loads per population equivalent (pe) per day of 16S rRNA, ARGs, MGEs, and the fecal indicator *E. coli* in function of the seasonal air temperature (panel A) and rainfall measured as Hydraulic load factor (HLF) (panel B).

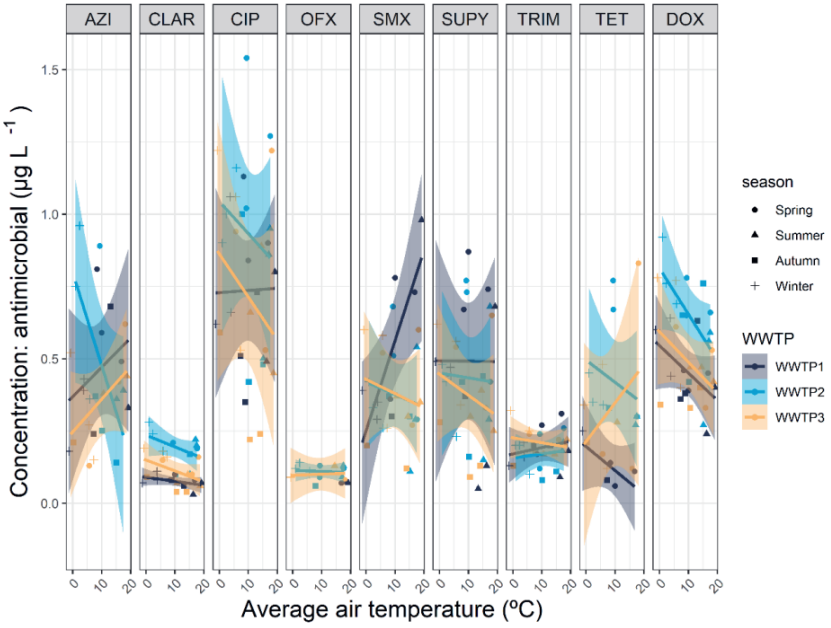


Figure S3.4. Influent concentrations of antimicrobials in function of seasonal air temperature

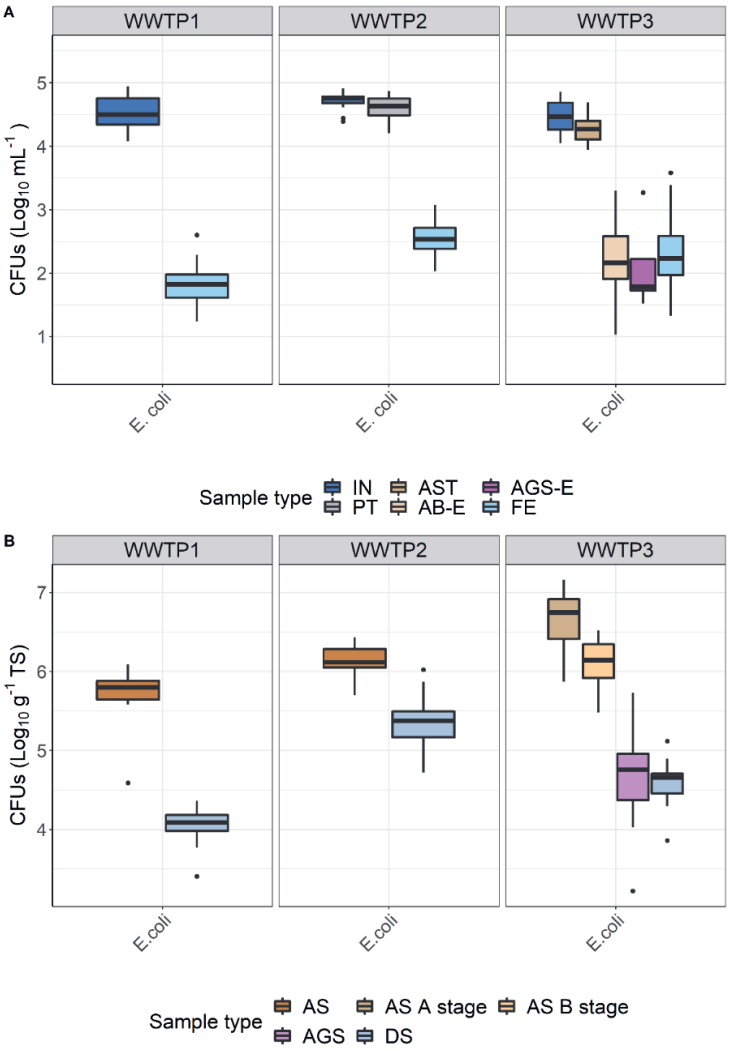


Figure S3.5. Prevalence of the fecal indicator *E. coli* in different samples of the waterline (A) and biosolids line (B) in three Dutch WWTPs through a year. The boxes represent the 2nd and 3rd quartiles. The middle line represents the median, and the whiskers represent 1st and 4th quartile. Single black dots represent outlier values. Sample's abbreviations: IN: Influent; PT: After primary treatment; AST: After A stage; AB-E: Effluent after AB treatment; AGS-E: Effluent after Aerobic granular sludge treatment; FE: Final Effluent; AS: Activated sludge; AS A stage: Activated sludge from the A stage of the AB system, AS B stage: Activated sludge from the B stage of the AB system; AGS: Aerobic granular sludge treatment; DS: Digested sludge

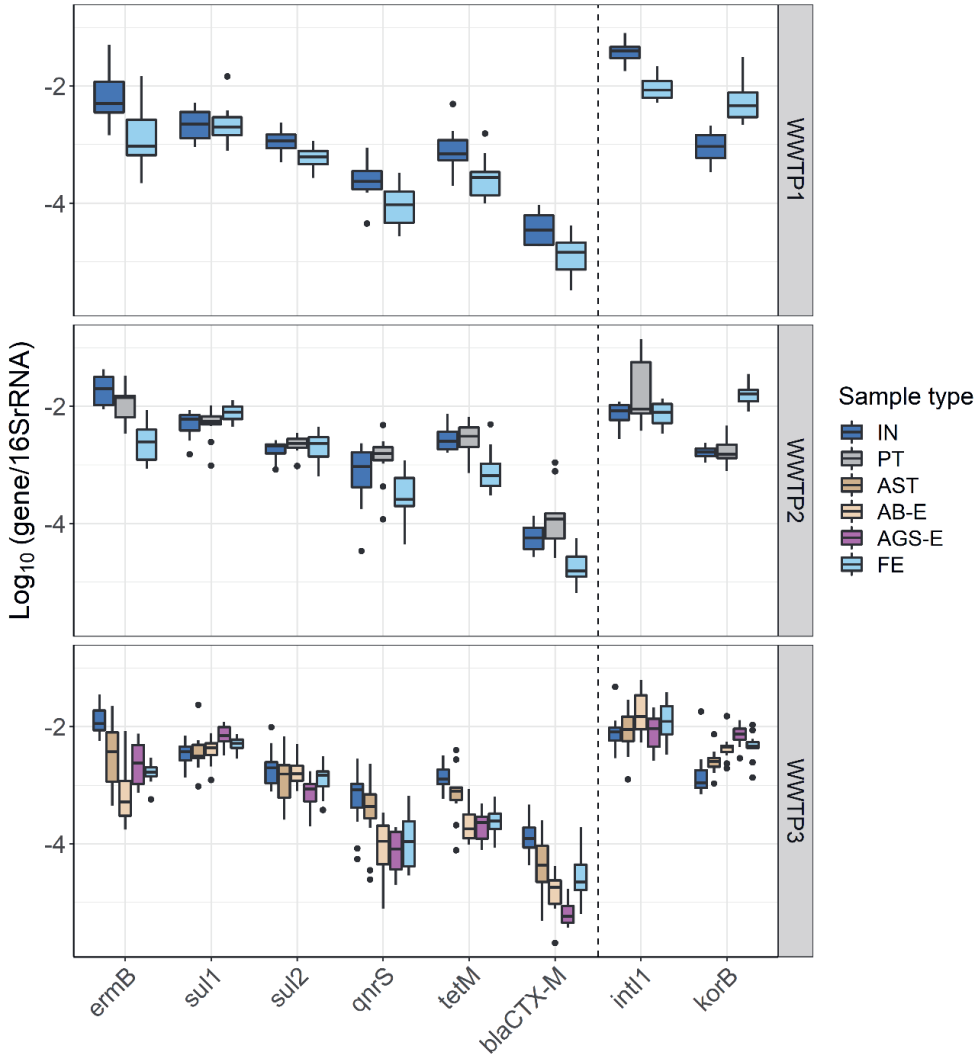


Figure S3.6. Relative abundance of ARGs and MGEs to 16S rRNA in the waterline of three Dutch WWTPs through a year. ARGs (right) and MGE(left) are separated for a better interpretation of the figure. The boxes represent the 2nd and 3rd quartiles. The middle line represents the median, and the whiskers represent 1st and 4th quartile. Single black dots represent outlier values. Sample's abbreviations: IN: Influent; PT: After primary treatment; AST: After A stage; AB-E: Effluent after AB treatment; AGS-E: Effluent after Aerobic Granular Sludge treatment; FE: Final Effluent;

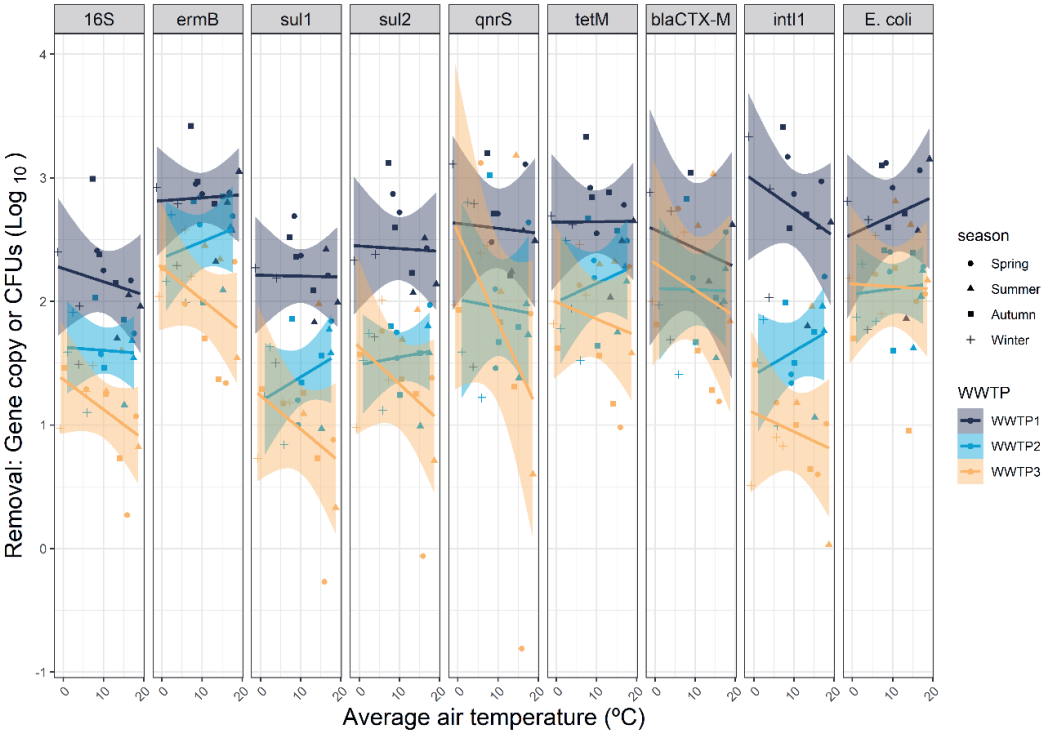


Figure S3.7. Removal of 16S, ARGs, MGEs and the fecal indicator *E. coli* in function of seasonal average seasonal air temperature

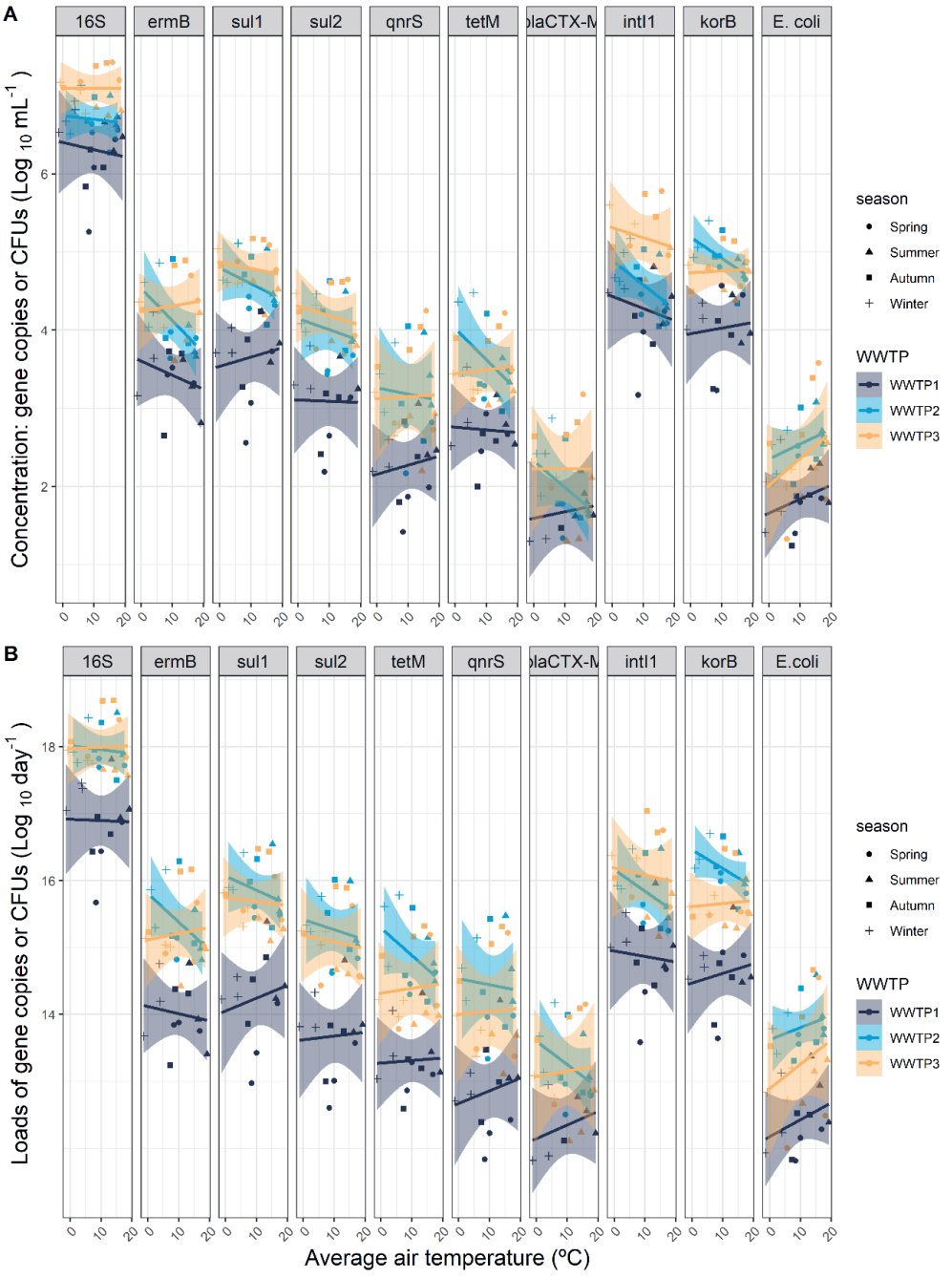


Figure S3.8. Effluent absolute concentrations of 16S rRNA, ARGs, MGEs, and *E. coli* (panel A) or daily loads (panel B) in function of seasonal average air temperature. In panel B, loads are used instead of concentration to account for the influence of flow and graphically observe only the variability caused by seasonal changes in temperature. Values corresponding to each of the four seasons are displayed with different symbols.

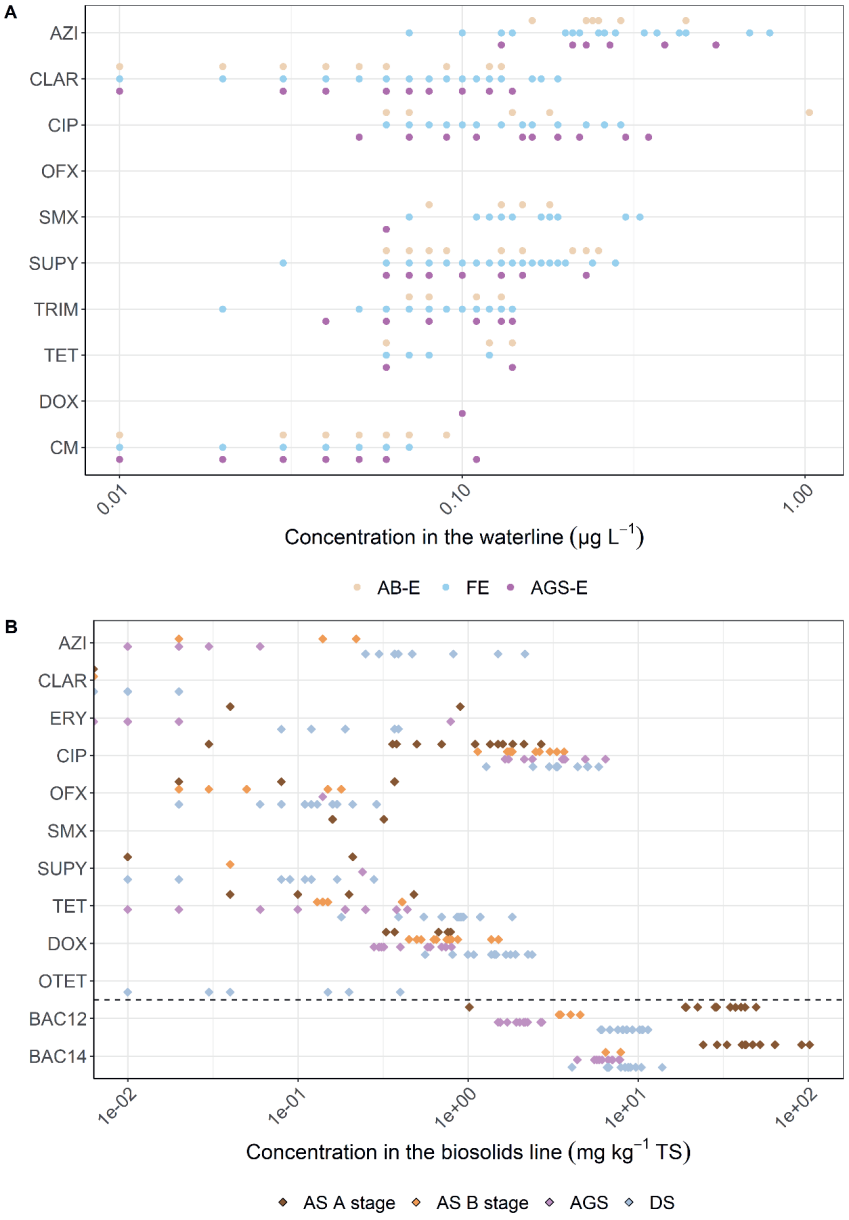


Figure S3.9. Antimicrobial residues in the waterline (panel A) and antimicrobial and disinfectant residues in the biosolids line (panel B) of WWTP3 over a year. Name of the compounds is presented in the y-axes and their respective concentration in each type of sample is represented in the x-axes. The concentrations of antimicrobials are expressed in $\log_{10}$ scale. Antimicrobials and disinfectant residues are separated by an horizontal dash line for a clearer interpretation of the figure.

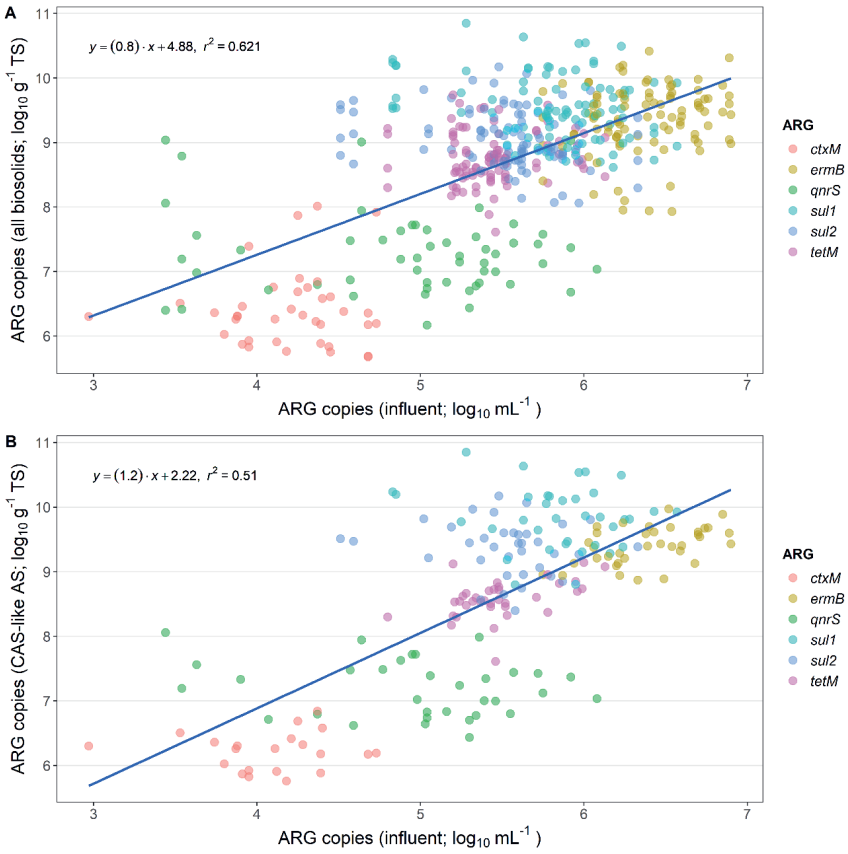


Figure S3.10.Linear relation of ARGs occurrence in influent line versus biosolids line (all biosolids samples) (panel A) and influent versus only CAS-Like Activated sludge samples (panel B). All biosolids samples include activated sludge samples, granular sludge samples, and digested sludge samples. CAS-Like samples include activated sludge samples from WWTP1, WWTP2, and B stage of WWTP3.

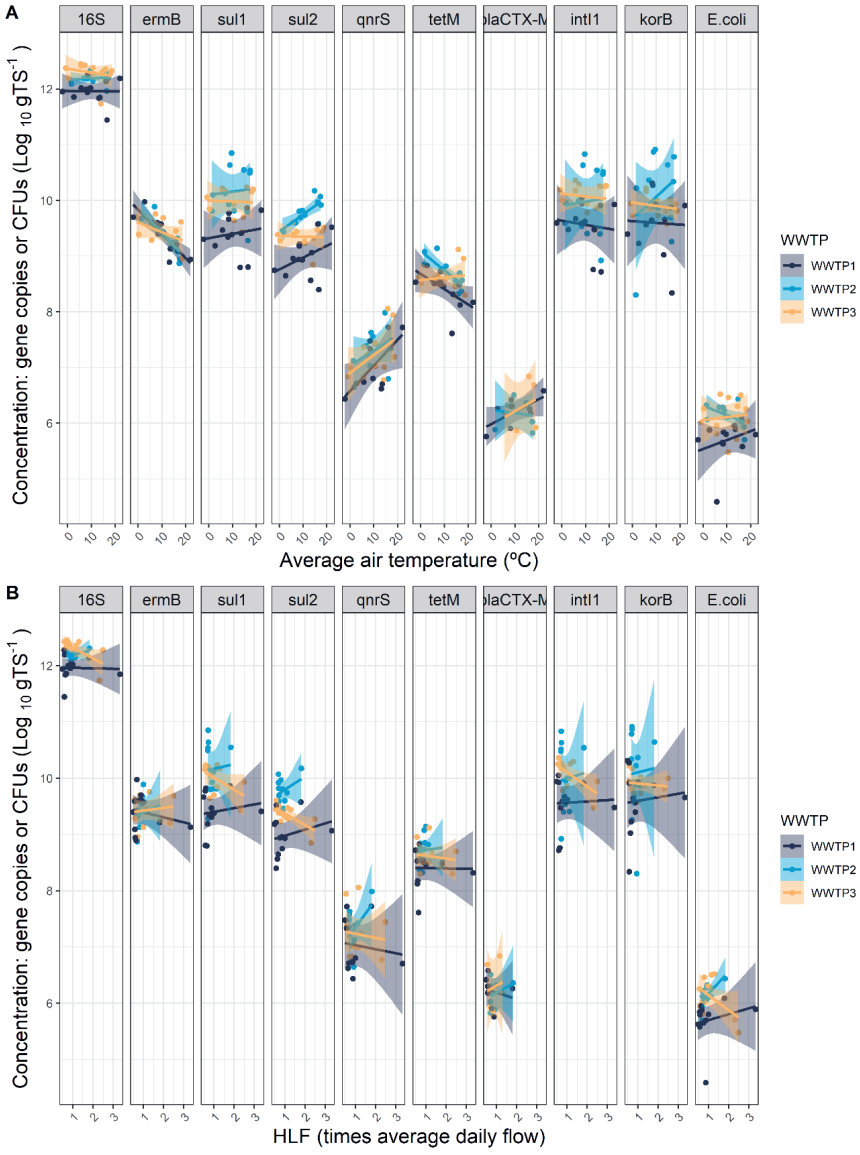


Figure S3.11. Absolute concentration of 16S rRNA, ARGs, MGEs, and *E. coli* in CAS-like Activated Sludge systems in function of the seasonal air temperature (panel A) and rainfall measured as hydraulic load factor (HLF) (panel B).

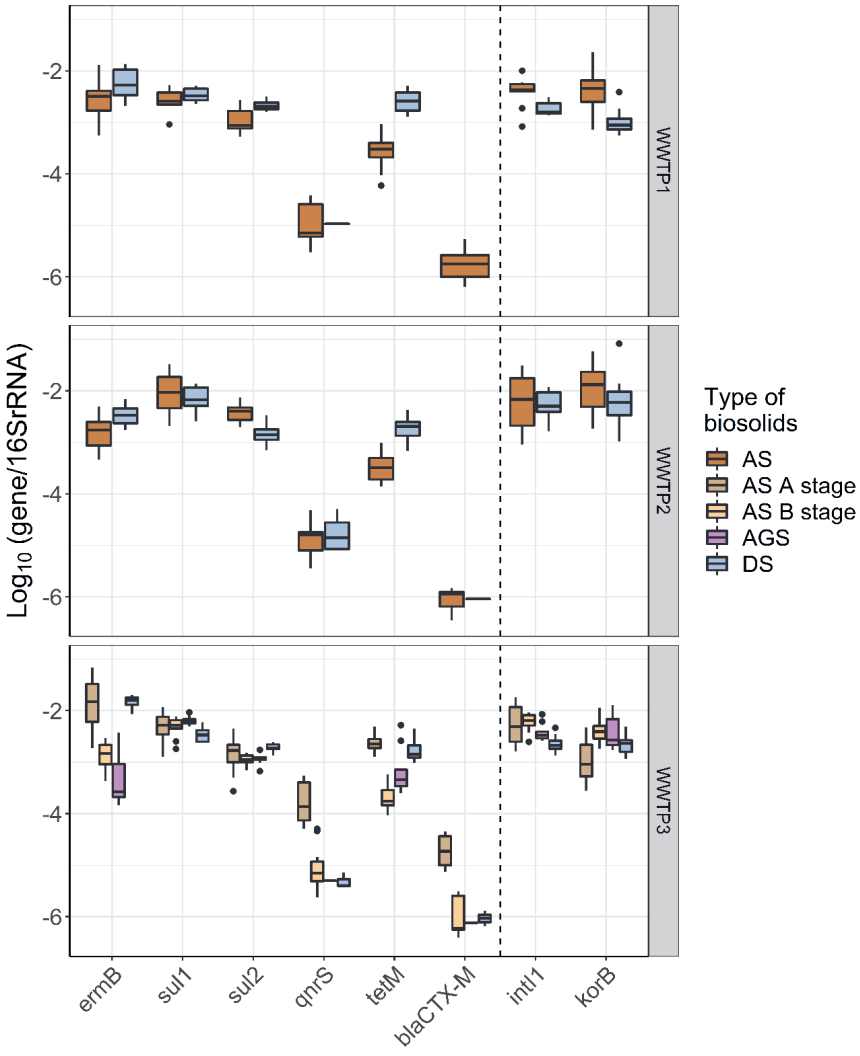


Figure S3.12. Relative abundance of ARGs and MGEs to 16S rRNA in the biosolids line of three Dutch WWTPs through a year. ARGs (right) and MGE (left) are separated for a better interpretation of the figure. The boxes represent the 2nd and 3rd quartiles. The middle line represents the median, and the whiskers represent 1st and 4th quartile. Single black dots represent outlier values. AS: Activated sludge; AS A stage: Activated sludge from the A stage of the AB system, AS B stage: Activated sludge from the B stage of the AB system; AGS: Aerobic granular sludge treatment; DS: Digested sludge.

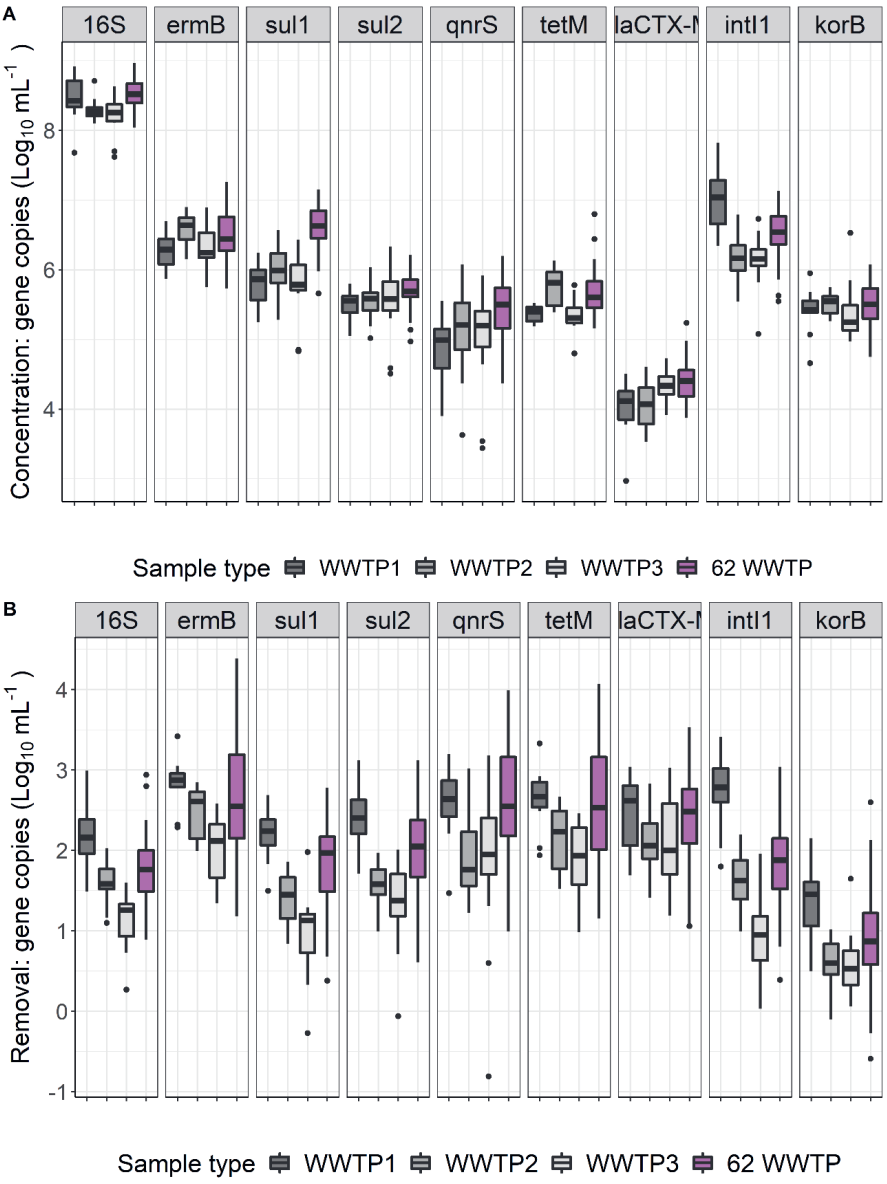


Figure S3.13. Variability in the influent concentration (panel A) and removal (panel B) of 16S rRNA, ARGs and MGEs, captured through a year of sampling in three WWTPs (grey scale) and single time points measurements in 62 WWTPs (magenta).

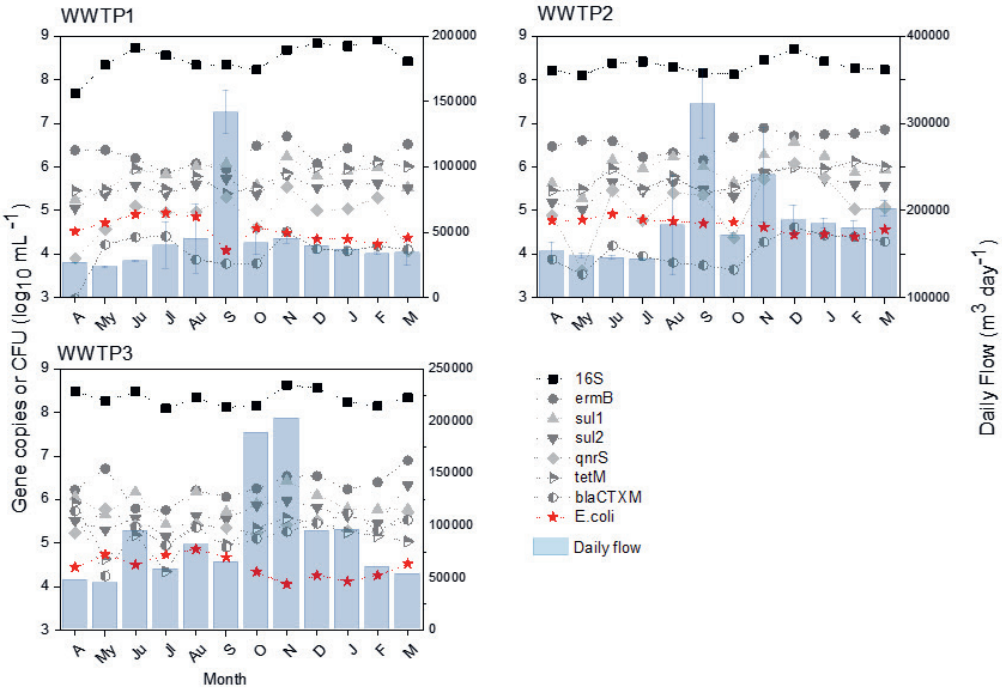


Figure S3.14 Temporal variation in the influent concentrations of ARGs, *E. coli*, and daily flow in a year.

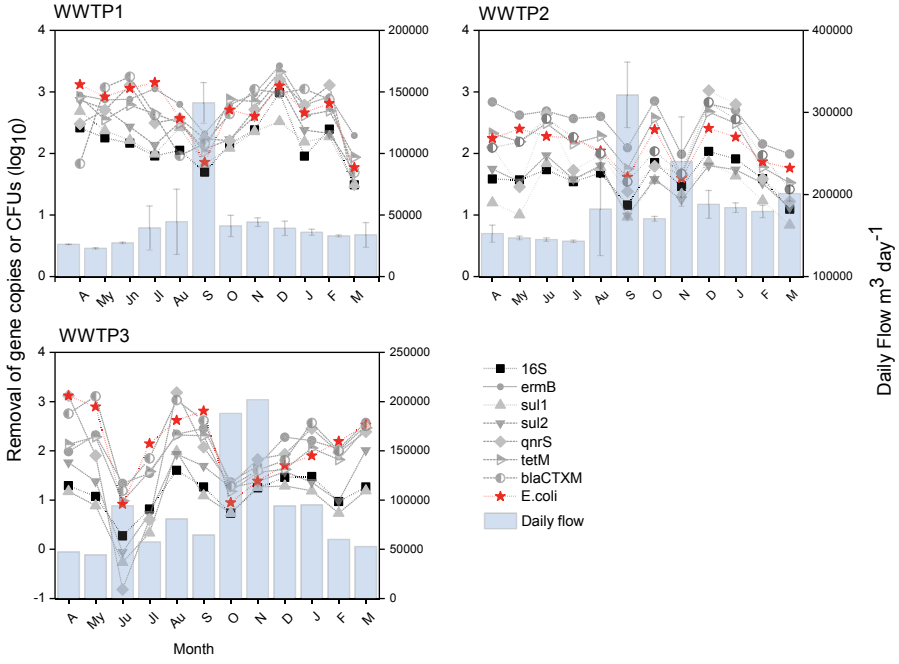


Figure S3.15. Monthly removal of 16S rRNA, ARGs and *E. coli*, and daily flow through a year.

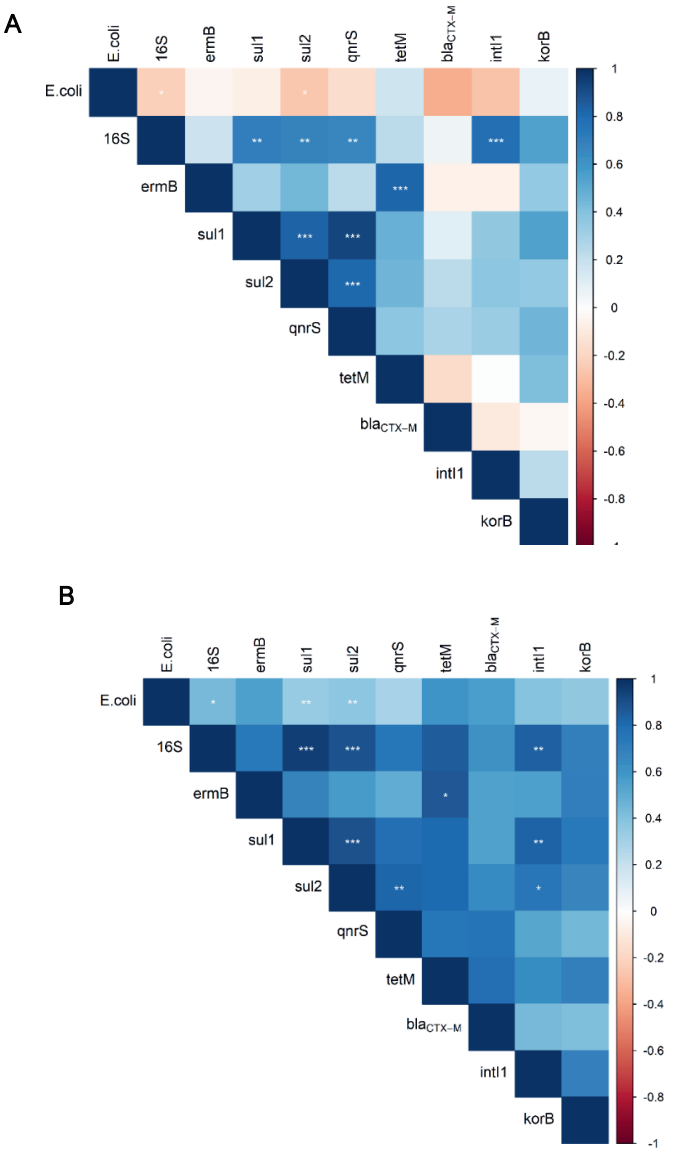


Figure S3.16. Correlation matrix (Pearson's correlation) of the absolute influent concentration (A) and removal efficiency (B) of the diverse ARGs, MGEs, and the proxies 16S rRNA gene and *E. coli*. Significant levels are * p<0.05; ** p<0.01; *** p<0.001.

3.10. References

Ali, M., Wang, Z., Salam, K.W., Hari, A.R., Pronk, M., Van Loosdrecht, M.C.M., Saikaly, P.E., 2019. Importance of species sorting and immigration on the bacterial assembly of different-sized aggregates in a full-scale Aerobic granular sludge plant. Environ. Sci. Technol. 53, 8291–8301. https://doi.org/10.1021/acs.est.8b07303

Auguet, O., Pijuan, M., Borrego, C.M., Rodriguez-Mozaz, S., Triadó-Margarit, X., Giustina, S.V. Della, Gutierrez, O., 2017. Sewers as potential reservoirs of antibiotic resistance. Sci. Total Environ. 605–606, 1047–1054. https://doi.org/10.1016/j.scitotenv.2017.06.153

Baran, W., Adamek, E., Ziemiańska, J., Sobczak, A., 2011. Effects of the presence of sulfonamides in the environment and their influence on human health. J. Hazard. Mater. 196, 1–15. https://doi.org/10.1016/j.jhazmat.2011.08.082

Barraud, O., Baclet, M.C., Denis, F., Ploy, M.C., 2010. Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. J. Antimicrob. Chemother. 65, 1642–1645. https://doi.org/10.1093/jac/dkq167

Barrios-Hernández, M.L., Pronk, M., Garcia, H., Boersma, A., Brdjanovic, D., van Loosdrecht, M.C.M., Hooijmans, C.M., 2020. Removal of bacterial and viral indicator organisms in full-scale aerobic granular sludge and conventional activated sludge systems. Water Res. X 6. https://doi.org/10.1016/j.wroa.2019.100040

Bates, D., Mächler, M., Bolker, B.M., Walker, S.C., 2016. Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Softw. 33, 251–264. https://doi.org/10.18637/jss.v067.i01

Bengtsson-Palme, J., Hammarén, R., Pal, C., Östman, M., Björlenius, B., Flach, C.F., Fick, J., Kristiansson, E., Tysklind, M., Larsson, D.G.J., 2016. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. Sci. Total Environ. 572, 697–712. https://doi.org/10.1016/j.scitotenv.2016.06.228

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ. Int. 86, 140–149. https://doi.org/10.1016/j.envint.2015.10.015

Cai, L., Ju, F., Zhang, T., 2014. Tracking human sewage microbiome in a municipal wastewater treatment plant. Appl. Microbiol. Biotechnol. 98, 3317–3326. https://doi.org/10.1007/s00253-013-5402-z

Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, P., Voolaid, V., Gurke, R., Virta, M., Berendonk, T.U., 2016. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. FEMS Microbiol. Ecol. 92, 1–10. https://doi.org/10.1093/femsec/fiw060

Clesceri, L.S., Rica, E.W., Baird, R.B., Eaton, A.D., 1998. No Title, in: Standard Methods for the Examination of Water and Wastewater. American Public Health Association, American Water Works Association and Water Environment Federation, Washington D.C.

Coutu, S., Wyrsch, V., Wynn, H.K., Rossi, L., Barry, D.A., 2013. Temporal dynamics of antibiotics in wastewater treatment plant influent. Sci. Total Environ. 458–460, 20–26. https://doi.org/10.1016/j.scitotenv.2013.04.017

De Graaff, M.S., Van Den Brand, T.P.H., Roest, K., Zandvoort, M.H., Duin, O., Van Loosdrecht, M.C.M., 2016. Full-Scale Highly-Loaded Wastewater Treatment Processes (A-Stage) to Increase Energy Production from Wastewater: Performance and Design Guidelines. Environ. Eng. Sci. 33, 571–577. https://doi.org/10.1089/ees.2016.0022

De Rooij, M.M.T., Hoek, G., Schmitt, H., Janse, I., Swart, A., Maassen, C.B.M., Schalk, M., Heederik, D.J.J., Wouters, I.M., 2019. Insights into Livestock-Related Microbial Concentrations in Air at Residential Level in a Livestock Dense Area. Environ. Sci. Technol. 53, 7746–7758. https://doi.org/10.1021/acs.est.8b07029

Di Cesare, A., Eckert, E.M., D'Urso, S., Bertoni, R., Gillan, D.C., Wattiez, R., Corno, G., 2016. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. Water Res. 94, 208–214. https://doi.org/10.1016/j.watres.2016.02.049

Dröge, M., Pühler, A., Selbitschka, W., 2000. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. Mol. Gen. Genet. 263, 471–482. https://doi.org/10.1007/s004380051191

Felis, E., Kalka, J., Sochacki, A., Kowalska, K., Bajkacz, S., Harnisz, M., Korzeniewska, E., 2020. Antimicrobial pharmaceuticals in the aquatic environment - occurrence and environmental implications. Eur. J. Pharmacol. 866, 172813. https://doi.org/10.1016/j.ejphar.2019.172813

Ferreira Da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O.C., Manaia, C.M., 2007. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. FEMS Microbiol. Ecol. 60, 166–176. https://doi.org/10.1111/j.1574-6941.2006.00268.x

Gao, P., Munir, M., and Xagoraraki, I. (2012). Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci. Total Environ. 421–422, 173–183. doi:10.1016/j.scitotenv.2012.01.061.

Ghosh, S., Ramsden, S.J., LaPara, T.M., 2009. The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. Appl. Microbiol. Biotechnol. 84, 791–796. https://doi.org/10.1007/s00253-009-2125-2

Gillings, M.R., Xuejun, D., Hardwick, S.A., Holley, M.P., Stokes, H.W., 2009. Gene cassettes encoding resistance to quaternary ammonium compounds: A role in the origin of clinical class 1 integrons? ISME J. 3, 209–215. https://doi.org/10.1038/ismej.2008.98

Göbel, A., Thomsen, A., McArdell, C.S., Joss, A., Giger, W., 2005. Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. Environ. Sci. Technol. 39, 3981–3989. https://doi.org/10.1021/es048550a

Gromaire, M.C., Garnaud, S., Saad, M., Chebbo, G., 2001. Contribution of different sources to the pollution of wet weather flows in combined sewers. Water Res. 35, 521–533. https://doi.org/10.1016/S0043-1354(00)00261-X

Gullberg, E., Albrecht, L.M., Karlsson, C., Sandegren, L., Andersson, D.I., 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. MBio 5, e01918-14. https://doi.org/10.1128/mBio.01918-14

Jechalke, S., Dealtry, S., Smalla, K., Heuer, H., 2013. Quantification of IncP-1 plasmid prevalence in environmental samples. Appl. Environ. Microbiol. 79, 1410–3. https://doi.org/10.1128/AEM.03728-12

Jiao, Y.N., Zhou, Z.C., Chen, T., Wei, Y.Y., Zheng, J., Gao, R.X., Chen, H., 2018. Biomarkers of antibiotic resistance genes during seasonal changes in wastewater treatment systems. Environ. Pollut. 234, 79–87. https://doi.org/10.1016/j.envpol.2017.11.048

Johnston, J., LaPara, T., Behrens, S., 2019. Composition and Dynamics of the Activated Sludge Microbiome during Seasonal Nitrification Failure. Sci. Rep. 9, 4565. https://doi.org/10.1038/s41598-019-40872-4

Jutkina, J., Rutgersson, C., Flach, C.F., Joakim Larsson, D.G., 2016. An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. Sci. Total Environ. 548–549, 131–138. https://doi.org/10.1016/j.scitotenv.2016.01.044

Karkman, A., Pärnänen, K., Larsson, D.G.J., 2019. Fecal pollution can explain antibiotic resistance gene abundances in anthropogenically impacted environments. Nat. Commun. 10, 1–8. https://doi.org/10.1038/s41467-018-07992-3

Karkman, A. Johnson, T.A., Lyra, C., Stedtfeld, R.D., Tamminen, M., Tiedje, J.M., Virta, M., 2016. High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. FEMS Microbiol. Ecol. 92. https://doi.org/10.1093/femsec/fiw014

Knapp, C.W., Zhang, W., Sturm, B.S.M., Graham, D.W., 2010. Differential fate of erythromycin and beta-lactam resistance genes from swine lagoon waste under different aquatic conditions. Environ. Pollut. 158, 1506–1512. https://doi.org/10.1016/j.envpol.2009.12.020

Korzeniewska, E., Harnisz, M., 2018. Relationship between modification of activated sludge wastewater treatment and changes in antibiotic resistance of bacteria. Sci. Total Environ. 639, 304–315. https://doi.org/10.1016/j.scitotenv.2018.05.165

Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2017. lmerTest Package: Tests in Linear Mixed Effects Models . J. Stat. Softw. 82. https://doi.org/10.18637/jss.v082.i13

Lépesová, K., Olejníková, P., Mackuľak, T., Tichý, J., Birošová, L., 2019. Annual changes in the occurrence of antibiotic-resistant coliform bacteria and enterococci in municipal wastewater. Environ. Sci. Pollut. Res. 26, 18470–18483. https://doi.org/10.1007/s11356-019-05240-9

Li, D.C., Gao, J.F., Zhang, S.J., Gao, Y.Q., Sun, L.X., 2020. Emergence and spread patterns of antibiotic resistance genes during two different aerobic granular sludge cultivation processes. Environ. Int. 137, 105540. https://doi.org/10.1016/j.envint.2020.105540

Lindberg, R.H., Wennberg, P., Johansson, M.I., Tysklind, M., Andersson, B.A.V., 2005. Screening of human antibiotic substances and determination of weekly mass flows in five sewage treatment plants in Sweden. Environ. Sci. Technol. 39, 3421–3429. https://doi.org/10.1021/es048143z

Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I.C., Zammit, I., Kampouris, I., Vaz-Moreira, I., Nunes, O.C., 2018. Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environ. Int. 115, 312–324. https://doi.org/10.1016/J.ENVINT.2018.03.044

Marti, E., Balcázar, J.L., 2013. Real-time PCR assays for quantification of qnr genes in environmental water samples and chicken feces. Appl. Environ. Microbiol. 79, 1743–1745. https://doi.org/10.1128/AEM.03409-12

Martínez-Carballo, E., González-Barreiro, C., Sitka, A., Kreuzinger, N., Scharf, S., Gans, O., 2007. Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part II. Application to sediment and sludge samples in Austria. Environ. Pollut. 146, 543–547. https://doi.org/10.1016/j.envpol.2006.07.016

Marx, C., Mühlbauer, V., Schubert, S., Oertel, R., Ahnert, M., Krebs, P., Kuehn, V., 2015. Representative input load of antibiotics to WWTPs: Predictive accuracy and determination of a required sampling quantity. Water Res. 76, 19–32. https://doi.org/10.1016/j.watres.2015.02.049

Mulder, I., Siemens, J., Sentek, V., Amelung, W., Smalla, K., Jechalke, S., 2018. Quaternary ammonium compounds in soil: implications for antibiotic resistance development. Rev. Environ. Sci. Biotechnol. 17, 159–185. https://doi.org/10.1007/s11157-017-9457-7

Novo, A., Manaia, C.M., 2010. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. Appl. Microbiol. Biotechnol. 87, 1157–1166. https://doi.org/10.1007/s00253-010-2583-6

Osińska, A., Korzeniewska, E., Harnisz, M., Felis, E., Bajkacz, S., Jachimowicz, P., Niestępski, S., Konopka, I., 2020. Small-scale wastewater treatment plants as a source of the dissemination of antibiotic resistance genes in the aquatic environment. J. Hazard. Mater. 381, 121221. https://doi.org/10.1016/j.jhazmat.2019.121221

Ottoson, J., Hansen, A., Björlenius, B., Norder, H., Stenström, T.A., 2006. Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. Water Res. 40, 1449–1457. https://doi.org/10.1016/j.watres.2006.01.039

Pallares-Vega, R., Blaak, H., van der Plaats, R., de Roda Husman, A.M., Hernandez Leal, L., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2019. Determinants of presence and removal of antibiotic resistance genes during WWTP treatment: A cross-sectional study. Water Res. 161, 319–328. https://doi.org/10.1016/j.watres.2019.05.100

Peak, N., Knapp, C.W., Yang, R.K., Hanfelt, M.M., Smith, M.S., Aga, D.S., Graham, D.W., 2007. Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. Environ. Microbiol. 9, 143–151. https://doi.org/10.1111/j.1462-2920.2006.01123.x

Pei, R., Kim, S.-C., Carlson, K.H., Pruden, A., 2006. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res. 40, 2427–2435. https://doi.org/10.1016/j.watres.2006.04.017

Pronk, M., de Kreuk, M.K., de Bruin, B., Kamminga, P., Kleerebezem, R., van Loosdrecht, M.C.M., 2015. Full scale performance of the aerobic granular sludge process for sewage treatment. Water Res. 84, 207–217. https://doi.org/10.1016/j.watres.2015.07.011

Quintela-Baluja, M., Abouelnaga, M., Romalde, J., Su, J.Q., Yu, Y., Gomez-Lopez, M., Smets, B., Zhu, Y.G., Graham, D.W., 2019. Spatial ecology of a wastewater network defines the antibiotic resistance genes in downstream receiving waters. Water Res. 162, 347–357. https://doi.org/10.1016/j.watres.2019.06.075

Rafraf, I.D., Lekunberri, I., Sànchez-Melsió, A., Aouni, M., Borrego, C.M., Balcázar, J.L., 2016. Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. Environ. Pollut. 219, 353–358. https://doi.org/10.1016/j.envpol.2016.10.062

Rahube, T.O., Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., Duenk, P., Lapen, D.R., Topp, E., 2014. Impact of fertilizing with raw or anaerobically digested sewage sludge on the abundance of antibiotic-resistant coliforms, antibiotic resistance genes, and pathogenic bacteria in soil and on vegetables at harvest. Appl. Environ. Microbiol. 80, 6898–6907. https://doi.org/10.1128/AEM.02389-14

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242. https://doi.org/10.1016/j.watres.2014.11.021

Rutgersson, C., Ebmeyer, S., Lassen, S.B., Karkman, A., Fick, J., Kristiansson, E., Brandt, K.K., Flach, C.F., Larsson, D.G.J., 2020. Long-term application of Swedish sewage sludge on farmland does not cause clear changes in the soil bacterial resistome. Environ. Int. 137, 105339. https://doi.org/10.1016/j.envint.2019.105339

Sabri, N.A., Schmitt, H., Van der Zaan, B., Gerritsen, H., Zuidema, T., Rijnaarts, H.H.., Langenhoff, A.A.M., 2018. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. J. Environ. Chem. Eng. https://doi.org/10.1016/j.jece.2018.03.004

Schages, L., Wichern, F., Kalscheuer, R., Bockmühl, D., 2020. Winter is coming – Impact of temperature on the variation of beta-lactamase and mcr genes in a wastewater treatment plant. Sci. Total Environ. 712, 136499. https://doi.org/10.1016/j.scitotenv.2020.136499

Shafrir, M., Avisar, D., 2012. Development method for extracting and analyzing antibiotic and hormone residues from treated wastewater sludge and composted biosolids. Water. Air. Soil Pollut. 223, 2571–2587. https://doi.org/10.1007/s11270-011-1049-5

Tong, J., Fang, P., Zhang, J., Wei, Y., Su, Y., Zhang, Y., 2019. Microbial community evolution and fate of antibiotic resistance genes during sludge treatment in two full-scale anaerobic digestion plants with thermal hydrolysis pretreatment. Bioresour. Technol. 288, 121575. https://doi.org/10.1016/j.biortech.2019.121575

Verburg, I., García-Cobos, S., Leal, L.H., Waar, K., Friedrich, A.W., Schmitt, H., 2019. Abundance and antimicrobial resistance of three bacterial species along a complete wastewater pathway. Microorganisms 7. https://doi.org/10.3390/microorganisms7090312

Zhang, C., Cui, F., Zeng, G. ming, Jiang, M., Yang, Z. zhu, Yu, Z. gang, Zhu, M. ying, Shen, L. qing, 2015. Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment. Sci. Total Environ. 518–519, 352–362. https://doi.org/10.1016/j.scitotenv.2015.03.007

CHAPTER 4

Temperature and nutrient limitations decrease the transfer of conjugative IncP-1 plasmid pKJK5 to wild *Escherichia coli* strains

Abstract

Plasmid-mediated dissemination of antibiotic resistance among fecal *Enterobacteriaceae* in natural ecosystems may contribute to the persistence of antibiotic resistance genes in anthropogenically-impacted environments. Plasmid transfer frequencies measured under laboratory conditions might overestimate plasmid transfer potential in natural ecosystems. This study assessed differences in the conjugative transfer of an IncP-1 plasmid (pKJK5) to three natural *E. coli* strains carrying extended-spectrum beta-lactamases, by filter mating. Matings were performed under optimal laboratory conditions (nutrient-rich LB medium and 37 °C) and environmentally relevant temperatures (25, 15 and 9 °C) or nutrient regimes mimicking environmental conditions and limitations (synthetic wastewater and soil extract). Under optimal nutrient conditions and temperature, two recipients yielded high transfer frequencies (5×10^{-1}) while the conjugation frequency of the third strain was 1000-fold lower. Decreasing mating temperatures to psychrophilic ranges led to lower transfer frequencies, albeit all three strains conjugated under all the tested temperatures. Low nutritive media caused significant decreases in transconjugants (-3 logs for synthetic wastewater; -6 logs for soil extract), where only one of the strains was able to produce detectable transconjugants. Collectively, this study highlights that despite less-than-optimal conditions, fecal organisms may transfer plasmids in the environment, but the transfer of pKJK5 plasmids between microorganisms is limited mainly by low nutrient conditions.

A modified version of this chapter has been published as: Pallares-Vega, R., Macedo, G., Brouwer, M.S.M., Hernandez Leal, L., van der Maas, P., van Loosdrecht, M.C.M., Weissbrodt, D.G., Heederik, D., Mevius, D., Schmitt, H., 2021. Temperature and Nutrient Limitations Decrease Transfer of Conjugative IncP-1 Plasmid pKJK5 to Wild *Escherichia coli* Strains. Front. Microbiol. 12. https://doi.org/10.3389/fmicb.2021.656250

4.1. Introduction

Antibiotic resistance is considered as one of the most significant challenges to global public health (Vinet and Zhedanov, 2010). The spread of antimicrobial resistance genes (ARGs) via horizontal gene transfer (HGT) between bacteria is a growing concern because it facilitates the dissemination of resistance across a wide variety of microorganisms. Understanding the dynamics of plasmid dissemination in the environment is fundamental to contain and mitigate antibiotic resistance challenge.

HGT is an effective ecological trait that shapes bacterial evolution (Ochman et al., 2000). Conjugative plasmids are relevant vectors for HGT (Smillie et al., 2010) and dissemination of antibiotic resistance (Carattoli, 2013). Gut bacteria from both animal and human origin comprise an important source of antibiotic resistant-conjugative plasmids (Ceccarelli et al., 2019; Hu et al., 2013). Gut bacteria are released into the environment through manure application to agricultural soils and wastewater discharges, ultimately resulting in the introduction of their ARGs, and plasmids in the environment. Despite having limited survivability, once introduced in the environment, gut bacteria might be able to transfer their resistance determinants to the natural bacterial community. *Escherichia coli* is widely accepted as a primary indicator of fecal contamination. Although most *E. coli* strains cause only mild infections, their presence is indicative of the potential presence of other more pathogenic organisms which may be relevant for human health.

Monitoring of environmental HGT remains challenging mainly due to cultivation bias (only 1% of indigenous bacteria are estimated to be cultivable (Amann et al., 1995)). Fluorescently labelled strains and plasmids comprise a promising methodology to study horizontal gene transfer in complex environments by culture-independent methods (Sørensen et al., 2005). Due to donor-recipient incompatibilities and detection limits of the methodology, the experimental design often requires a compromise to guarantee the detection of transconjugants (Pinilla-Redondo et al., 2018; Sørensen et al., 2005). As a result, studies addressing environmental dissemination of resistant plasmids usually apply conditions that are optimal for bacterial transmission, namely high bacterial densities, optimal growth temperatures, and/or high nutrient availability (Bellanger et al., 2014a; Jacquiod et al., 2017). Although being relevant for specific scenarios such as mesophilic anaerobic digesters, greenhouses or wastewater in low latitude countries (Al Qarni et al., 2016; Fan et al., 2019), these settings do not reflect the usual average conditions of manured soils, water bodies and wastewater (Abis and Mara, 2006; Barrios-

Hernández et al., 2020; Osińska et al., 2020). Such discrepancies in the experimental design might lead to an overestimation of plasmid transfer frequencies and dissemination potential in the environment. Therefore, better insights into how environmental parameters affect plasmid transfer are needed.

The aim of this study was to evaluate *in vitro* the role of environmental factors that could potentially hamper conjugative plasmid transfer from gut bacteria once discharged into the environment. A conjugative broad host range IncP-1 plasmid (pKJK5) was used as vector. IncP-1 plasmids have comparatively high conjugation rates and thus allow for analysis of conjugation frequency also under suboptimal conjugation conditions. In addition, IncP-1 plasmids often carry clinically relevant ARGs (Rozwandowicz et al., 2018), are abundant in (waste)water (Pallares-Vega et al., 2021), manure (Binh et al., 2008), and soil environments (Shintani et al., 2020) and can potentially disseminate among a wide diversity of phylogenetic groups (Popowska and Krawczyk-Balska, 2013). Furthermore, IncP-1 plasmids (i.e. RP4, pB10 and pKJK5) comprise the predominant plasmids in studies addressing transfer events in environmental settings (Bellanger et al., 2014b; Inoue et al., 2005; Klümper et al., 2015; Li et al., 2018a). Solid-surface filter matings were conducted to study HGT between *Escherichia coli* strains (as both donor and recipients), with animal *E. coli* strains harbouring extended-spectrum beta-lactamase genes on known plasmid types as recipients, representative of *E. coli* introduced with animal manure. The transfer was evaluated under different (i) donor-to-recipient cell proportions, (ii) mating temperatures, or (iii) nutritional compositions. The criteria to select the used conditions was based on the presumable main abiotic challenges that gut bacteria face when discharged into the environment, namely nutrient limitations and close-to psychrophilic conditions. The donor-to-recipient cell proportions were tested to assess the limit of the system while aiming for a natural proportion of donor and recipient cells in the mating. By using the same species and a broad-host-range plasmid, potential host-vector and interspecies incompatibilities were discarded as factors. *E. coli* was chosen as a model system for bacteria of public health relevance that can potentially move between anthropogenic related and natural environments, and it was hypothesized that lower temperatures and lower nutrient concentrations would limit plasmid transfer.

4.2. Materials and methods

4.2.1. Selection and characterization of strains and plasmids

Three extended-spectrum beta-lactamase (ESBL) carrying *E. coli* strains (09.54, 38.27, and 39.62) isolated from fecal samples of calves or poultry were used as recipients during the mating experiments (**Table 4.1**). These strains were part of a database from the Dutch national veterinarian institute (Wageningen Bioveterinary Research, WBVR), studying the prevalence of ESBLs in plasmids. The strains qualify for this work because of their species, diverse plasmid content, and because they had been sequenced under the scope of WBVR projects. A genetically engineered *E. coli* strain previously described by Klümper et al. (2015) was selected as donor for the broad-host-range plasmid of the incompatibility group IncP-1. The donor strain (*E. coli* K-12 MG1655::*lacI*^{q}*-pLpp-mCherry-Km*^{R}) is commonly used in dual-labelling fluorescence reporter-gene approaches coupled with fluorescence-activated cell sorting (Pinilla-Redondo et al., 2018) due to the conditionally expressible green fluorescent proteins (GFP) in its IncP-1 plasmid (pKJK5). The IncP-1 plasmid carries a kanamycin resistance determinant and lacI^{q} repressible promoter upstream the *gfpmut3* gene (Bahl et al., 2007; Klümper et al., 2015a; Sengeløv et al., 2001).

Table 4.1. Bacterial strains of *E. coli* used as donor and recipient of broad-host-range IncP-1 plasmid, and their characteristics. ST: Sequence Type. AMP: Ampicillin, CTX: Cefotaxime, KM: Kanamycin, SMX: Sulfamethoxazole, TET: Tetracycline, TMP: Trimethoprim.

Agent	ST	Role	Origin	Resistance profile	Plasmids	Source
E. coli **MG1655::lacIq-pLpp-mCherry-Km^{R}**	ST10/ ST262	Donor	Laboratory strain	AMP, SMX,KM, TMP.	pKJK5:: PA1/04/03-gfpmut3 (IncP-1)	(Klümper et al., 2015a)
E. coli **09.54**	ST21/ ST481	Recipient	Veal calf	AMP, CTX, SMX, TETR	IncK	This study
E. coli **38.27**	ST10/ ST2	Recipient	Poultry	AMP, CTX, SMXR, TET	IncFI, IncH1, IncI1, p0111	This study
E. coli **39.62**	ST101 / ST88	Recipient	Poultry	AMP, CTX, SMX, TET	IncFIB/FII IncK	This study

In order to fully characterize the strains, whole-genome sequencing using paired-end Illumina was performed, as previously described by Rozwandowicz et al. (2020). The annotation of the sequences was achieved with Prokka version 1.12 (Seemann, 2014). The corresponding sequence type was conducted with the Multi Locus Sequence Typing online tool MLST 2.0

(Larsen et al., 2012), using the two available schemes (Jaureguy et al., 2008; Wirth et al., 2006). For typing the donor strain and relate the natural recipient strains to the donor, a reference sequence of *E. coli* MG1665 (accession number: NC_000913.3) from GenBank was used. The existence of plasmid replicons within the strains was analyzed with PlasmidFinder (Carattoli et al., 2014), applying an identity cut-off equal or greater than 98%. The annotated sequences are deposited in GenBank, BioProject PRJNA661180 under the accession no. JADPVO000000000 (09.54), JADPVP000000000 (38.27) and JADPVQ000000000 (39.62). A core and accessory genome analysis of the donor and recipient strains was conducted with Roary version 13.0 (Page et al., 2015) in Galaxy version 21.01 (https://usegalaxy.eu/). A maximum likelihood tree based on nucleotide sequence was built with FastTree version 2.1.10 (Price et al., 2010) in Galaxy and graphic visualization of the core and accessory genome was achieved with Phandango (Hadfield et al., 2018).

To identify suitable selective conditions for the identification of transconjugants, the antimicrobial susceptibility profile for each strain was determined by disc diffusion test, according to EUCAST guidelines (EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing - version 6.0; available at https://www.eucast.org/). The results were interpreted based on the EUCAST-defined Breakpoints tables for interpretation of MICs and zone diameters (version 8.0) and are summarized in **Table S4.1** in Supplementary Information. **Figure 4.1** displays this study's schematic of the experimental design and procedure.

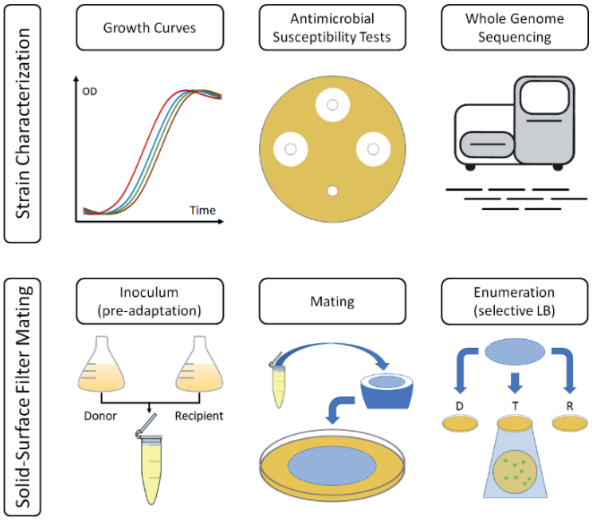


Figure 4.1 Overview of the procedure to quantify transconjugants. Donors and recipients were grown separately before being mixed, filtered, and incubated for 2 hours, at different temperatures or at different media. Bacteria were recovered, and enumerated, in LB containing antibiotic combinations specific for donors, recipients, or transconjugants

4.2.2. Culture media and growth curves

Luria-Bertani (LB), synthetic wastewater (SWW), and soil extract (SE) were used as culture media for the filter matings. Pure bacterial cultures were prepared and maintained in LB broth or plates (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, sodium chloride 5 g L^{-1}, and agar 15 g L^{-1}) prior to the experiments, and for the selection of donor, recipients, and transconjugants after the matings, the LB plates were enriched with kanamycin (100 µg mL^{-1}; Sigma Aldrich), tetracycline (16 µg mL^{-1}; Sigma Aldrich), and both kanamycin and tetracycline (100 and 16 µg mL^{-1}), respectively.

The SWW aimed to mimic the average conditions and nutrient proportions of conventional domestic wastewater. The composition was based on that of Boeije et al. (1999), and ISO 11733 guideline and adjusted to a theoretical COD:N:P concentration and molar ratio close to that of Dutch wastewater (100 : 9.1 : 1.4, **Supplementary information Table S4.2**). The SWW solution contained 0.07 g L^{-1} urea, 0.011 g L^{-1} NH_4Cl, 0.015 g L^{-1} peptone P (Oxoid, UK), 0.015 g L^{-1} Lab Lemco (Oxoid, UK), 0.05 g L^{-1} starch, 0.04 g L^{-1} glycerol that was sterilized by autoclaving. After sterilization, the mix was completed with 0.25 g L^{-1} sodium acetate, 0.12 g L^{-1} skimmed milk powder (Sigma Aldrich, NL), 0.05 g L^{-1} glucose, 0.025 g L^{-1} $FeSO_4$, 0.005 $CaCl_2$ g L^{-1}, 0.025 g L^{-1} $NaHCO_3$ and 0.02 g L^{-1} $MgHPO_4{\cdot}3H_2O$, 0.016 g L^{-1} L $K_3PO_4{\cdot}H_2O$ (unless indicated otherwise, the components were purchased at VWR, NL). These solutions were separately autoclaved or filter-sterilized prior to their aseptic addition to the final solution. SWW media was finally supplemented with the addition of 0.1% (v/v) of trace metal solution which contained 0.280 g L^{-1} NaEDTA, 0.180 g L^{-1} $ZnCl_2$, 1.144 g L^{-1} H_3BO_3, 0.025 g L^{-1} $CoCl_2{\cdot}6H_2O$, 0.589 g L^{-1} $MnCl_2{\cdot}2H_2O$, 0.120 $CuCl_2{\cdot}2H_2O$, 0.068 g L^{-1} $NiCl_2{\cdot}6H_2O$, 0.025 g L^{-1} $Na_2MoO4{\cdot}5H_2O$, 0.212 g L^{-1} $KCr(SO_4)_2{\cdot}12H_2O$. The pH was adjusted with NaOH 1M to match the values found in wastewater (6.8 ± 0.1). When needed, agar (15 g L^{-1}) was added for solid media preparation.

Soil samples for SE medium preparation were collected in the late fall of 2019, from a local dairy farm (Friesland, Netherlands) that uses the field for pasture (grassland) and had not been recently subjected to manure application. In total, 7 kg of sandy loam soil were collected from the field and homogenized. The collected soil was air-dried for three days and stored in 500 g zip bags at 4°C until being used. The SE media was prepared as described by Musovic et al. (2010). Briefly, 500 g of dried soil was mixed with 500 mL of demineralized water. Then, the mixture was shaken horizontally for 3 hours and left for passive settling of the particles for 5 hours. After the 5 hours, the supernatant was pipetted and autoclaved (for 15 minutes, at 121

°C) and stored at 4 °C, up to one month. The pH values were not adjusted and were kept at its original values (5.0 – 5.3), and no buffer solutions were used to maintain the pH in the different culture media because they could introduce potential nutrients (e.g., phosphate). When needed, agar was added as aforementioned.

The general chemical compositions of the LB, SWW, and SE media were determined by ion chromatography (IC), and inductively coupled plasma (ICP-OES). The determination of the chemical oxygen demand (COD), and the total nitrogen was achieved with commercially available kits (LCK 514 and LCK 338; Hach). The determination of the total organic carbon (TOC) was achieved with Shimadzu TOC-L_{CPH} analyzer. The composition of the different media used is displayed in **Table 4.2.**

Table 4.2. Media composition of the culture media used in the matings with either Luria-Bertani (LB), synthetic wastewater (SWW) or soil extract (SE) medium. Legend: total organic carbon (TOC), chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP)

(mg L^{-1})	LB		SWW		SE	
Compound	Mean	SD	Mean	SD	Mean	SD
TOC	6,820	80	219	1.0	45	-
COD	21,450	2,450	529	37	173	1
TN	2,050	20	48	2	7	0.4
TP	151	1	7.2	0.2	4	0.0
Ca^{2+}	9	1	3.6	0.0	104	1
K^{+}	272	2	11.5	0.1	21	9.9
Mg^{2+}	7	0.1	37.6	0.1	5	0.6
$Fe^{2+/3+}$	0	0.0	4.5	0.0	<0.05	-
S	127	0	38	1	67	2
NH_4^{+}	60	0	6.2	0.0	<0.10	-
NO_3^{-}	4	0.0	<0.10	-	10	0.0
PO_4^{3-}	259	1	>20	-	12	0.0
SO_4^{2-}	96	9	11	0	191	7

To quantify the effect of the temperature change in the growth, an inoculum volume of 0.2 % (final volume) of an overnight culture of each strain was transferred to fresh LB, and incubated at 9, 15, 25, or 37 °C. The Pathogen Modelling Program (PMP) online model (available at: https://pmp.errc.ars.usda.gov/default.aspx) was used to predict the incubation time range to measure bacterial density. To determine the effect of the nutrient composition, inoculums of 0.2 % (final volume) overnight culture of each strain were transferred to SWW or SE media, and monitored up to three days. The optical density at 600 nm (OD_{600}), was measured in a UV-Vis Spectrophotometer (Shimadzu Corp). Colony forming units (CFUs) were determined after preparing 10-fold serial dilutions with saline solution (NaCl; 0.85 %), plating in LB agar, and incubating at 37 °C, overnight. Measurements were performed in biological triplicates.

4.2.3.Solid surface filter matings: Standard conditions

Conjugation is a process that requires cell proximity and stable spatial conditions during the mating time (ca 3-5 min). Although these conditions can occur in the liquid phase, they are more likely in "surface-like" configurations (Zhong et al., 2010) occurring in soil grains, sludge flocs or biofilms. Bearing this in mind, filter mating was chosen to study the plasmid transfer.

The conjugation assays were performed by mixing 150 µL of fresh culture of the donor and recipient, and vacuum filtered through mixed-cellulose ester filters (0.45 µm; Millipore) in a Millipore filtration system. Prior to mixing, the cultures were grown for approximately 3 h in LB at 37ºC to achieve a density of 10^8 CFU mL^{-1}, as experimentally defined by the growth curves. After filtration, the mixed cultures were transferred to plates containing LB and cells were then incubated at 37 °C. Following the incubation period, the cells were detached from the filter by vortexing in 1 mL of sterile LB broth, for 5 min. Subsequently, serial decimal dilutions were prepared in sterile saline solution, and 100 µL was spread on LB plates containing kanamycin (donors), tetracycline (recipients) and a combination of both (transconjugants). The results were observed after a 24-h incubation period (total counts), at 37 °C, and another 24-h incubation period (coloured colonies), at 4 °C. The incubation at 4 °C was performed to enhance the visualization of the GFP protein (Scott et al., 2006) and to count the green colonies, the plates were observed in a blue-light transilluminator (Safe Imager™ 2.0; Invitrogen). To confirm the validity of each assay, matings with only the donor or the recipient were also performed. Each mating was performed in biological triplicates on alternative days.

4.2.4. Solid surface filter matings: Modified conditions

When different proportions of donor-to-recipient ratios (D/R) were tested, the donor cultures harvested until 10^8 CFU mL^{-1} were serially diluted (10 and 100-fold) in LB and 150 µL was mixed with 150 µL recipient culture to reach the corresponding ratios D/R of 1:10 and 1:100. A total volume of 200 µL of the mixtures were then filtered, and the mating and incubation were performed as aforementioned. The approximate cell density in the filters was 8.9 x 10^6 CFU cm^{-2}. The effect of temperature in transfer frequency was assessed by following the standard condition procedure but incubating the filters at 25, 15 and 9 °C in LB plates pre-conditioned to the corresponding temperatures. To assess the influence of nutrient availability in the transfer frequency, matings conducted in SWW and SE media were compared to standard nutrient-rich media LB. For SWW matings, donor and recipient cell cultures were pre-adapted to low nutrient conditions by growing them in SWW media (1% overnight inoculum) for approximately 4 h with 180 rpm agitation until a cell density of 10^8 CFU mL-1 was achieved

(**Figure S4.4**). Then, cell cultures were mixed and filtered as aforementioned in the standard conditions, and filters were placed in SWW agar plates. Plates were incubated at 37 °C for 2 h. For SE matings, no pre-growth from donor nor recipients could be obtained in SE broth, as indicated by the corresponding growth curves (data not shown). Instead, late log phase LB cultures of both donor and recipients ~10^9 were centrifuged and washed twice in saline solution, and the pellet was finally resuspended in 10 mL of SE broth and incubated overnight at 37 °C. Before incubation, an aliquot of the resuspended cells was serially diluted in saline solution, plated in LB and incubated overnight at 37 °C. Following the incubation and based on the cell counts of the suspensions, the cell density of both donor and recipient SE cultures were adjusted to approximately 10^8 CFU mL^{-1}, mixed in 1:1 ratio and filtered as indicated in the standard procedure. Filters were then placed on SE media and incubated at 37 °C for 24 h. In all modified filter matings, cell recovery and subsequent plating were performed as mentioned in the standard conditions.

4.2.5. Genetic characterization of donor, recipient and transconjugants

To confirm the strain identity (donor, recipient and transconjugants), five to ten isolates per mating were collected randomly from each of the media containing the antibiotics, and PCR was performed on the crude cell extracts. Reactions targeting the 16S rRNA gene, mCherry, and *gfpmut3* were prepared in 25-µL reactions containing PCR buffer (1x), (Invitrogen, NL) $MgCl_2$ (3.0 mM), (Invitrogen, NL), dNTPs (0.2 mM) (Promega, NL), forward and reverse primers (0.4 µM; **Table S4.3**), *Taq* polymerase (1.25 U) (Invitrogen, NL), and 1 µL of DNA. The PCR reactions were carried out in a T100 Thermal Cycler (BioRad), following similar denaturation conditions (95 °C for 30 s), but specific annealing and elongation conditions (57, 55, or 60 °C for 30 s; and 30 – 90 s at 72 °C for the 16S rRNA, *gfpmut3*, and mCherry genes, respectively), in 30 cycles. The specificity of the PCR products was confirmed by visualization in 1.5 % agarose gel stained with ethidium bromide.

4.2.6. Data Analysis

One-way analysis of variance (ANOVA) was conducted to detect differences in the conjugation frequencies between strains, temperatures, and culture media. The ANOVA tests were followed by TukeyHSD post-hoc analysis, and homogeneity of variance was confirmed with Levene's test. Data normality was confirmed with Shapiro-Wilk's method, and when normality was not achieved, group comparison was performed using the equivalent non-parametric test (Kruskal-Wallis). A significance score of $p < 0.05$ was considered to be statistically relevant. These analyses were performed with R version 3.5.1 (R Core Team, 2018)

and RStudio (Version 1.1.456; https://www.rstudio.com/). Used software packages consisted of *reshape* (Wickham, 2007) and *tidyverse* (Wickham et al., 2019), a set of packages designed for data cleaning, trimming, and visualization; of *Rcmdr* (Fox, 2005), *PMCMRplus* (Thorsten, 2020), and *car* (Fox and Weisberg, 2019) for ANOVA and Levene's test.

4.3. Results

4.3.1. Effect of donor-to-recipient (D/R) ratios

Before the temperature and nutrients assays, the D/R ratios were tested to assess the limit of the system while aiming for a natural proportion of donor and recipient cells in the mating.

Under optimal conditions and 1:1 D/R ratio (37 °C and LB, 8.9 x 10^6 CFU cm^{-2}), two out of three *E. coli* strains (38.27 and 39.62) yielded high transconjugant numbers (10^9 CFUs mL^{-1}) and transfer frequency (5 x 10^{-1}) of IncP-1 plasmids. On the other hand, the mating with strain 09.54 produced 10^6 CFU mL^{-1} (transfer frequency of 10^{-3}). The transfer frequency, measured as the transconjugants-to-donors ratio (T/D), resulted in a slight increase in the 1:10 and 1:100 D/R proportions in comparison with the 1:1 proportion in all strains (except for one replicate of strain 09.54; **Figure 4.2**). Contrarily, the transconjugants-to-recipients ratio (T/R) decreased with the different D/R ratios, approximately -0.7 logs and -1.8 logs in the 1:10 and 1:100 proportions, respectively (strains 38.27 and 39.62). A stronger effect of D/R was observed for strain 09.54, where the T/R decreased 1-3 logs and 3-4 logs in the 1:10 and 1:100 proportion, respectively. Similar results were found for the absolute numbers of transconjugants (Figure S1 in Supplementary information). No transconjugants were recovered for one replicate in the mating of the strains 09.54 (1:100; Figure S1). At both 1:10 and 1:100 proportions, transconjugant numbers reached approximately 10^3 CFUs mL^{-1} for at least one of the replicates, which was close to the detection limit (10^2 CFUs mL^{-1}).

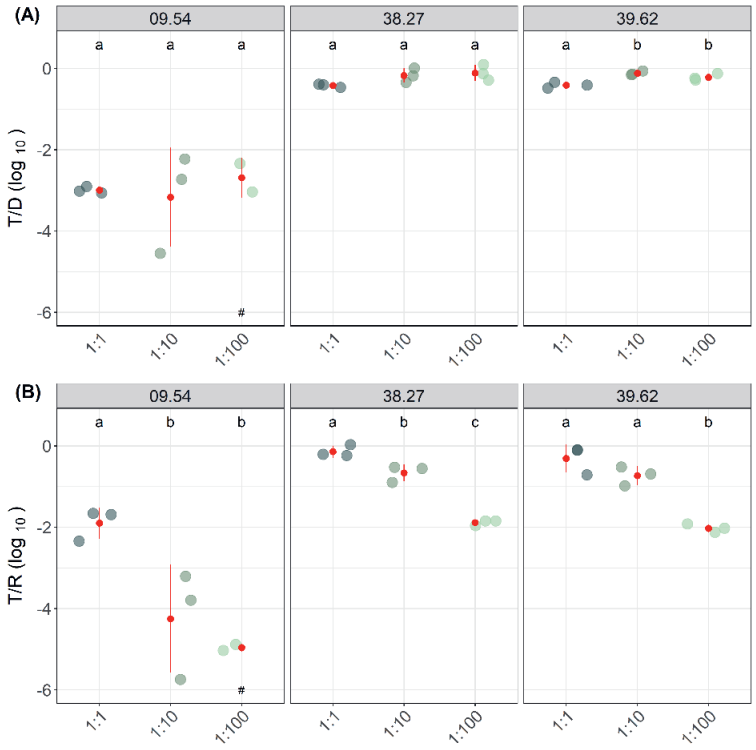


Figure 4.2. Donor-to-recipient proportions had significant effects on plasmid transfer. Depending on the indicator and strain used, the donor concentration increased or decreased, the transfer frequency. Relative counts of transconjugant-to-donor (T/D; A) and transconjugant-to-recipient (T/R; B) ratios, after 2-h matings performed at three donor-to-recipient ratios (1:1, 1:10, 1:100) are shown together with average and standard deviation values (in red). Different colors depict distinct donor-to-recipient ratios. [a, b, c] Indicate significantly different groups in the transfer frequency between ratios (PostHoc Tukey test, $p < 0.05$), and replicates with no detected transconjugants are highlighted ([#]).

4.3.2. Role of temperature on conjugative transfer

Conjugation efficiency among ESBL *E. coli* strains was assessed at temperatures ranging from the optimal laboratory (37 °C), room (25 °C) and relevant environmental (15, 9 °C) conditions.

Overall, lower temperatures significantly reduced the number of conjugation events ($p < 0.01$; **Figure 4.3**). Both T/D and T/R decreased with decreasing temperatures, with a more pronounced reduction in strain 09.54 than in the other two strains (**Figure 4.3**). The highest number of transconjugants was obtained at 37 °C, and at 25 °C, and the number of transconjugants decreased roughly 1 log (strains 38.27 and 39.62) or 2 logs (strain 09.54), depending on the strain. With further temperature reduction, lower transconjugant numbers were observed, and at 9 °C, conjugation still occurred in all tested strains.

The lowest number of transconjugants was obtained at 9 °C for strains 38.27 and 39.62. In strain 09.54, the minimum transconjugant number was already reached at 15 °C and maintained at 9 °C. However, higher variability among replicates was noticeable with strain 09.54 (**Figure S4.2**), and one replicate did not yield detectable transconjugants (**Figure S4.2**).

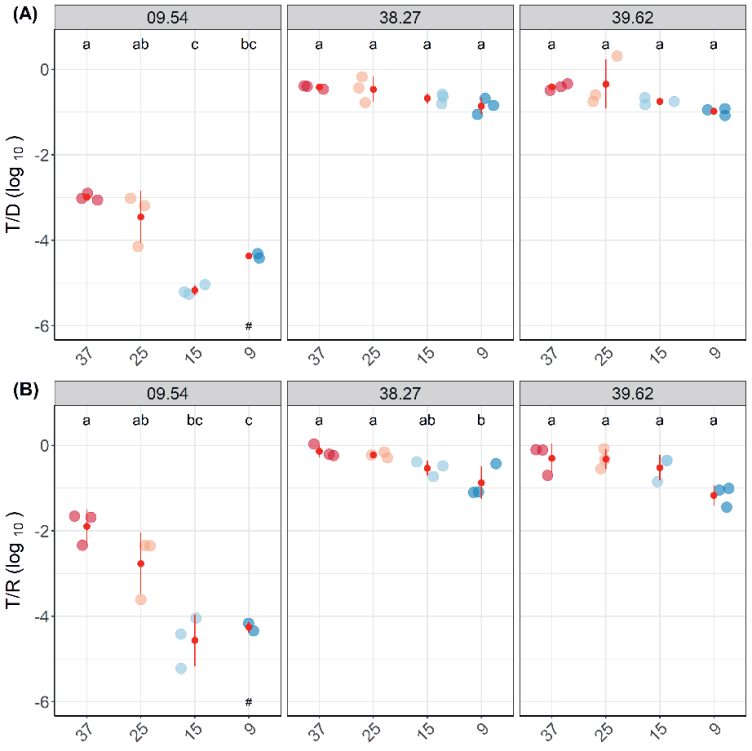


Figure 4.3 Lower temperature reduced the number of conjugation events. Relative counts of transconjugant-to-donor (T/D; A) and transconjugant-to-recipient (T/R; B) after 2h-matings performed, at diverse temperatures (37 – 9 °C), are shown together with average and standard deviation values (in red). Different colors depict distinct temperatures. [a, b, c] Indicate significantly different groups in the transfer frequency between temperatures (PostHoc Tukey test, $p < 0.05$), and replicates with no detected transconjugants are highlighted ([#]).

4.3.3. Role of nutrient concentrations on conjugative transfer

Differences in plasmid transfer under diverse nutrient regimes were assessed by comparing conjugation yields and transfer frequencies between rich nutrient media (LB) and common surrogates for natural conditions such as SWW and SE media.

In all tested strains, the decrease in the nutrient concentration of the media resulted in a substantial decrease in conjugation events (**Figure 4.4**). In comparison with the matings performed in LB, SWW reduced conjugation events by roughly 2 logs. In SE, a 4-log reduction

was observed for strain 39.62 (compared to LB), but no transconjugants were recovered for other strains, despite several attempts.

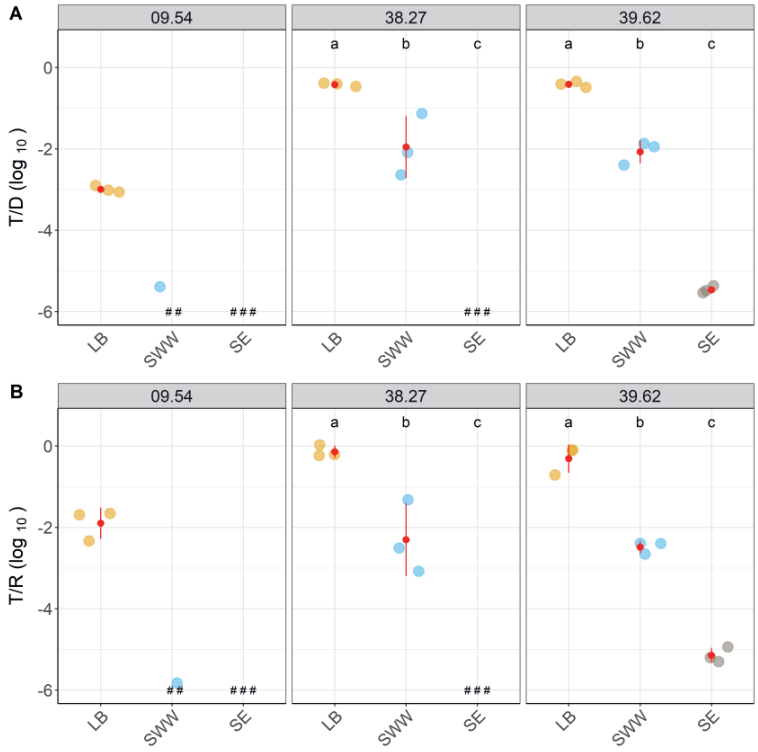


Figure 4.4. Decrease in nutrient concentration reduced conjugation events. Relative counts of transconjugant-to-donor (T/D; A) and transconjugant-to-recipient (T/R; B) after 2h-matings performed, at diverse nutrient conditions (Luria-Bertani, LB; synthetic wastewater, SWW; and soil extract, SE), are shown together with average and standard deviation values (in red). The replicates with no detected transconjugants are highlighted (#). Different colors depict distinct media. [a, b, c] Indicate significantly different groups of transfer frequency between culture media (PostHoc Tukey test, $p < 0.05$), and replicates with no detected transconjugants are highlighted (#).

The decline in transconjugant numbers was particularly severe for strain 09.54, which presented the lower number of transconjugants in LB. Its transconjugants were only recovered in one out of three matings performed in SWW, and when SE was used, a further decrease in the number of transconjugants was observed. While matings with strain 39.62 yielded 1.3 x 10^3 CFUs mL^{-1} transconjugants (3 and 6 logs lower than in SWW and LB, respectively; **Figure S4.3**), the strains 09.54 and 38.27 did not produce detectable transconjugants (**Figure S4.3**).

4.4. Discussion

The effects of temperature and nutrient abundance during mating of an IncP-1 plasmid were evaluated in three natural ESBL *E. coli* recipient strains by monitoring both total amount of transconjugants and transfer frequencies. The results confirmed that psychrophilic temperatures during mating, as well as nutrient limitation, resulted in the reduction of transfer events. The decrease in the number of transconjugants was more prominent with lower nutrients than with lower temperatures.

4.4.1. Transfer efficiency varied across strains

Under optimal physiological conditions for the growth of the three *E. coli* strains 09.54, 38.27 and 39.62 tested (rich LB medium, higher mesophilic temperature of 37 °C), the conjugative transfer of plasmid significantly differed among the recipients. Two strains showed a high frequency of transfer (5×10^{-1}) while the third (strain 09.54) had 2 logs less. High frequency of transfer is common among IncP-1 plasmids (Thomas and Smith, 1987), which are naturally derepressed (Bradley et al., 1980). Similar transfer frequencies (10^{-2}) have been described before for the pKJK5 plasmid in soil microcosms (Musovic et al., 2006). The difference of transfer frequency among strains from the same species can relate to strain-specific characteristics that either avoid or limits the expression of the new acquire genes in the recipient cell (Frost and Koraimann, 2010). The plasmid stability and replication depend heavily on complex coordination and synchronicity between the vector and host (Novick, 1987). In the present study, only one bacterial species (*E. coli*) was used to minimize potential genetic incompatibilities between donor and recipients. However, even when the same species are used, variable transfer frequencies are often reported. For instance, Dimitriu et al. (2019) observed a difference up to 5 orders of magnitude in the transfer frequencies of an IncF and IncP-1 among naturally co-occurring *E. coli* isolates. These significant differences are likely linked to the genetic diversity within species. Here, the accessory genes in the used strains corresponded to roughly 50% of the genomic content (**Figure S4.5**). However, which of these accessory traits can be the cause of variation remains a matter of discussion. Dimitriu et al. (2019) found no preferential transfer among isolates sharing serotype or closely related phylogeny. Instead, they proposed that conjugal transfer was favoured by clone-relationship, derived from similar restriction-modification systems. Contrarily, a recent study evaluating the transfer of ESBL plasmids among clinical *E. coli* isolates could not find such a relationship Benz et al. (2021).

In addition to host-recipient dynamics, plasmid to plasmid interactions could also affect the transfer dynamics. The stability of a newly acquired plasmid can be strongly influenced by the presence of other plasmids inside the cell (i.e. incompatibility). Here, we prevented the possible incompatibility issues by using strains with plasmids belonging to distinct incompatibility groups. Still, alternative effects of co-resident plasmids have been proposed recently. Enhanced transfer frequency of IncP-1 plasmids towards recipient cells hosting IncF plasmids has been observed (Gama et al., 2017). Although the mechanism of action is not entirely clear, the authors suggest that it is not a cooperative process but rather opportunistic use of the IncF transfer machinery by IncP-1 plasmids (Gama et al., 2017). In our experiments, we observed that the two strains with higher transfer frequency contained natural IncF plasmids (among others), whereas 09.54 harboured an IncK plasmid. However, further analysis would be necessary to confirm the role of the co-existing plasmid in the recipient cell.

4.4.2. Reducing input of donors reduced overall transfer frequency

A lower D/R proportion resulted in a decreased number of transconjugants, suggesting that the relative proportion of donors to recipients can limit HGT.

Receiving environmental compartments typically contain high cell densities, for instance, activated sludge usually contains between 10^9- 10^{10} CFU mL^{-1} (Manti et al., 2008) and topsoil (the first 10-15 cm) contain between 10^{14} – 10^{15} cells/m^3 (Bickel and Or, 2020). However, exogenous bacteria that enter the system (potential donors) might not be as numerous. For example, assuming a soil density of 1.5, it results in having 10^8 – 10^9 cells/g soil, while the manure from cattle and pigs contains roughly 10^5 *E. coli* cells g^{-1} (Schmitt et al., 2019), at least a 1,000-fold difference. This means that the proportion of potential donors is quite small, considering the receiving community. This proportion may depend on multiple factors, including sewage flows or manure application rates, but it is reasonable to expect that the potential donors will be a minority in the compartment to which they were introduced.

During conjugation assays, high cell densities (8.9 x 10^6 CFU cm^{-2}) would mirror natural systems. Conversely, the use of D/R ratios lower than 1:1 (i.e., 1:10 and 1:100) would presumably reflect more accurately the conditions found in anthropogenically impacted environments. However, to observe differences in conjugation rates under varied conditions, the number of donors should be sufficient to produce a detectable amount of transconjugants with a wide margin from the limit of detection (3 to 4 logs) in the matings performed under optimal conditions. Goodman et al. (1993) and Rochelle et al. (1989) observed that a minimum

of 10^4 CFU cm^{-2} of donors and recipients were necessary to observe transconjugants. Here, conjugation occurred at donor densities as low as 10^4 CFU cm^{-2} yielding a high amount of transconjugants (10^8) for two of the strains (38.27 and 39.62), but not for the third one (strain 09.54). For this last strain, transconjugants were undetectable or close to the limit of detection with initial donor densities of 10^4 or 10^5 CFU cm^{-2} (D/R of 1:100 and 1:10, respectively) Considering that low D/R could prevent the monitoring of conjugation events for at least one of the strains, the subsequent experiments were conducted with a D/R ratio of 1:1. Similar cell densities and ratios have been previously advised to observe changes in conjugal transfer across a range of (presumably) unfavourable conditions (Fernandez-Astorga et al., 1992).

4.4.3. Lower temperature inhibited plasmid transfer but not entirely

The highest number of transconjugants was obtained at 37 °C, which is also the optimal growth temperature for *E. coli*. However, growth of donors and recipients was observed between their concentrations at the start of the experiment and in the controls (approximately 1 log, in all strains; **Figure S4.2**). Together with growth curve data, this suggests that, at 37 °C, part of the transconjugant numbers originated from clonal expansion rather than a new transfer event. Conversely, at other temperatures, the amount of transconjugants observed reflected more accurately the real number of conjugation events, as the 2-h mating time concurred with the lag phase, and, consequently, clonal expansion can assume to be negligible.

Fluctuations in temperature are known to greatly affect the growth and metabolic functions of microorganisms (Trevors et al., 2012). Yet, the effect of a wide range of temperatures on conjugative antibiotic resistance-related plasmids has seldom been addressed (Bale et al., 1988; Banerjee et al., 2016; Inoue et al., 2005). Although cold conditions are predominantly found around the planet (Rodrigues and Tiedje, 2008) and in relevant environments for antibiotic resistance spread (**Table S4.4**), studies addressing the environmental dissemination of antibiotic resistant plasmids in microcosms often used rather warm (>25 °C) settings. Warm temperatures (25-30 °C) are also typical for *in vitro* studies that focus on either capturing environmental plasmids or addressing the microbial community permissiveness of a given plasmid because high conjugation rates are required for detecting a high diversity of transconjugants (Jacquiod et al., 2017; Li et al., 2020; Li et al., 2018a).

Conjugation occurred at environmental temperatures (i.e., 15 °C), which are average temperatures found in wastewater and soil worldwide (**Table S4.4**), but it also occurred at 9 °C. Typically, most wastewater treatment plants do not operate at temperatures below 9 °C

(because of nitrification failure), but in some countries, particularly northern countries, they can operate at temperatures close to 0 °C (Delatolla et al., 2012; Hoang et al., 2014). The use of different strains emphasized that the effect of temperature on the transfer frequency is recipient-dependent, and probably not affected just by chromosomally-encoded factors but also by resident plasmids in the recipient. The different outcomes observed between strains highlight the difficulty of inferring results applied to all putative recipient strains, even when they belong to the same species.

4.4.4. Lower nutrient composition hindered conjugation

A stronger effect on the transfer frequency was observed in matings performed with lower nutrient concentrations, where the frequency of conjugation was proportional to the nutrient richness of the culture media (LB > SWW > SE). In some cases, it was not possible to recover transconjugants in SE. Some authors suggest that plasmid transfer is related to cell growth and does not occur in non-growing cells (Kohyama and Suzuki, 2019; Seoane et al., 2011). Others consider that it happens after cell division and right before entering a non-growing phase (Headd and Bradford, 2020). We observed conjugation in SE media for at least one of the conjugation pairs, although cell growth was not observed for either donor or recipients in this media.

Comparatively, the SE and SWW media used in this study contained 40 to 300-fold (SE), and 20- to 40-fold (SWW) lower basic nutrients (C, N, and P) concentrations than the classical nutrient-rich media (LB; Table 2). Conjugation requires energy and cellular resources to occur, and thus, one could expect that low nutrient conditions would hamper plasmid transfer (Goodman et al., 1993). Interestingly, the effect of nutrient deprivation on conjugation is seldom documented. Fernandez-Astorga et al. (1992) addressed the effect of available TOC in liquid media, finding transconjugants even at 1 mg L^{-1} of TOC. Inoue et al. (2005) observed decreasing transconjugants in media with a decreasing amount of dissolved organic carbon (DOC) (6'636 to 21.6 mg L^{-1}), including LB, synthetic, and real wastewater. However, in the two aforementioned studies and elsewhere (Grabow et al., 1975a; Headd and Bradford, 2018; MacDonald et al., 1992; O'Morchoe et al., 1988), donor and recipient cells were pre-grown in a nutrient-rich media and then subjected to conjugation in the low nutrient media. Extra energy and nutrients stored in the cells during this pre-growth phase may allow bacteria to undergo conjugation in an earlier stage of the mating, potentially masking the effect of lower nutrition conditions on conjugation (Curtiss et al., 1969). To bypass this bias, Goodman et al. (1993) starved donors and recipients in minimal media (low amount of salts and no carbon source)

prior to the conjugation. They found that, despite the lack of nutrients, conjugation occurred after the donors were starved up to 3 or 20 days, when *E. coli* or *Vibrio* sp. were the donors, respectively. In the current study, when addressing conjugal transfer in low nutrient media, cells were also pre-incubated in the corresponding low-nutrient media (SWW or SE) to avoid the influence of intracellular nutrient reservoirs

Then again, carbon concentration is likely not the only nutrient that can limit conjugation. In their work, Inoue et al. (2005) observed that transconjugants and transfer rates were 2.5 logs higher in SWW than in 16-fold diluted LB, while both contained similar DOC content (410 mg L^{-1}). Possibly, a higher concentration of other nutrients (nitrogen, phosphorus or specific cations) in the SWW allowed an increase in conjugation frequencies and/or clonal expansion of the transconjugants. Pre-growth in media lacking casamino acids delayed *pili* formation after restoring nutritional conditions (Curtiss et al., 1969). As *pili* formation is protein-dependent, N-compounds are required for plasmid transfer. Despite being an essential nutrient, the role of phosphate or inorganic phosphorus deprivation in conjugation has not been explored yet. Phosphorus is known to be a limiting factor of cell growth and metabolism in oligotrophic environments (Smith and Prairie, 2004). In *E. coli*, phosphorus starvation induces a wide range of metabolic changes, including cell surface modification and increase of cell adhesion characteristics (adhesins and fimbria), which could affect the interaction between cells and ultimately the conjugation rates. Finally, the concentration of other micronutrients as divalent cations might also influence conjugation. Recently, Sakuda et al. (2018) observed that the addition of divalent cations to low nutrient media (Ca^{2+} and Mg^{2+}) increased the conjugation frequency of IncP-7 plasmids among *Pseudomonas* strains. Yet, the molecular mechanisms of this effect remain unclear.

Moreover, in the present study, the pH values of the different media were not maintained or adjusted, except in SWW. In SWW, the pH was adjusted to 6.8, close to the ones observed in wastewater (6.5-8.5 (Prot et al., 2020)) while the pH from SE was kept at its original value (5.0 – 5.3), which was representative of Dutch soils of this texture (Oenema, O., Römkens, 2004). Soil was kept at ambient pH to maintain the solubility of soil nutrients. As pH can affect bacterial growth, it could have also contributed to the decrease of transconjugants in this study observed for soil. Indeed, it has been shown that pH values in this range (5.0 – 5.3) can decrease conjugation (Richaume et al., 1989), but it only resulted in a maximum of 3-fold reduction (0.5 logs) when compared to conjugation occurring at neutral pH. In the context of the present study, it is difficult to discriminate what was the effective contribution of pH in

decreasing plasmid transfer in SE. However, given the several log decrease in transconjugants, it is reasonable to say that the lower nutrient content had a more important contribution in SE.

4.4.5. Extrapolation of the results and limitations of the study.

This study addresses the influence of temperature and nutrient conditions on a specific system based on *E. coli* strains and an IncP-1 broad-host range plasmid. Likely, the impact of the factors addressed here would differ per species. Bacteria better suited to thrive under typical environmental conditions will most likely be less affected by low temperatures and nutrient conditions, as observed by a longer ability (+13 days) for conjugal transfer when using pre-starved *Vibrio* spp. as donor instead of *E. coli* (Goodman et al., 1993). In addition, the plasmid characteristics (e.g. size, incompatibility group) obviously determine absolute transfer rates. Thus, further research addressing other combinations of donors-recipients will be desirable.

4.5. Conclusions

When moving from laboratory conditions to environmentally relevant conditions for soils and WWTPs, both lower temperature and lower nutrient concentrations showed to reduce the conjugal transfer of an IncP-1 plasmid significantly. The effect of lower nutrient concentrations on the number of transconjugants was stronger than the effect of lower temperatures. While nutritional conditions appear critical, the role of single nutrients, such as N and P, is not entirely clear and deserves further follow-up research. Furthermore, the transfer potential was recipient-dependent and varied within ESBL *E. coli* strains of the same species.

To conclude, although abiotic factors can hamper plasmid transfer, measurable conjugation between *E. coli* still occurred under conditions that mimicked those commonly found in the wastewater and soil environment (9 – 25 °C). Despite conjugation being observed between strains of the same species, this study shows that fecal indicator bacteria were capable of donating plasmids in less-than-optimal contexts, and consequently, can be a source of transferable antibiotic resistance traits once they reach the environment.

4.6. Acknowledgements

This work was performed in the cooperation framework of Wetsus, European Centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces.

This work has also received funding from STOWA and from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie [grant agreement No. 665874]. The authors like to thank the participants of the Wetsus research theme Source Separated Sanitation for the fruitful discussions and their financial support.

The authors gratefully acknowledge the support of the former students Xavier Gallego y van Seijen, Hana Piková, Gonzalo Monteoliva, Wesley Klaassen, Amaya Gómez, Dimitrios Maroulis, and Jenneke van der Draai for their relentless technical assistance in performing the conjugation essays. We thank Professor Søren J. Sørensen for kindly donating the donor strain.

4.7. Supplementary Information

Table S4.1. Antibiotic susceptibility determined by disc diffusion test, according to EUCAST guidelines. Strains are classified in Resistant (R), Susceptible (S) or Intermediate resistance (I).

Strain	Ampicillin AMP (10 ug)	Cefotaxime CTX (5 ug)	Ciprofloxacin CIP (5 ug)	Gentamicin GEN (10 ug)	Sulfamethoxazole SMX (25 ug)	Tetracycline TET (30 ug)	Kanamycin KN (30 ug)	Rifampicin RF (5 ug)
E. coli MG1655::*lacI*^{q}-*pLpp-mCherry-Km*^{R} / pKJK5::$P_{A1/04/03}$-*gfpmut3*	R	S	S	S	R	S	R	R
09.54	R	R	S	S	R	R	I	R
39.62	R	R	S	S	R	R	S	R
38.27	R	R	S	S	R	R	I	R

Table S4.2. Average annual concentrations of organic matter (COD, BOD), total nitrogen (TN) and total phosphorus (TP) in the influent wastewater of Dutch WWTPs between 2000-2018. Source CBS: https://opendata.cbs.nl/statline/#/CBS/nl/dataset/7477/table?dl=3DD6. Abbreviations: pe– population equivalents, COD – Chemical Oxygen Demand; BOD – Biological Oxygen Demand, na: data not available., SD: Standard deviation,

Year	Number of WWTPs	Total capacity (x1.000 pe)	COD (mg L^{-1})	BOD (mg L^{-1})	TN (mg L^{-1})	TP (mg L^{-1})
2000	391	na	470	180	43	7
2001	384	na	461	175	42	7
2002	378	na	477	185	44	7
2003	378	na	550	213	51	9
2004	375	na	506	194	46	8
2005	368	na	525	198	48	8
2006	363	na	520	196	48	8
2007	356	na	471	174	44	7
2008	351	na	503	192	47	8
2009	351	na	536	208	49	8
2010	349	30,365	513	200	46	7
2011	346	30,383	526	206	48	7
2012	343	30,358	505	199	46	7
2013	341	30,364	520	209	48	7
2014	337	30,237	548	218	50	7
2015	334	30,246	516	209	45	7
2016	327	30,122	541	225	49	7
2017	326	29,904	546	232	49	7
2018	323	29,942	593	248	54	7
		Average	517	203	47	7.4
		SD	32	19	3	0.6
		COD:N:P ratio	100		9.1	1.4

Table S4.3. Primers used for PCR confirmation of strains.

Target	Primer name	Sequence (5´- 3´)	Amplicon size (bp)	Reference
16S rRNA	27F	AGA GTT TGA TCC TGG CTC AG	1465	(Frank et al., 2008)
	1492R	GGT TAC CTT GTT ACG ACT T		
gfpmut3	q_GFPmut3-FW	TCG GTT ATG GTG TTC AAT GC	146	(Norman et al., 2014)
	q_GFPmut3-RV	GAC TTC AGC ACG TGT CTT GTA G		
mCherry	q_mCherry-FW	CCC CGT AAT GCA GAA GAA GA	99	Eurofins Genomics (Vidgren and Gibson, 2018)
	q_mCherry-RV	TTC AGC CTC TGC TTG ATC TC		

Table S4.4. Temperature range in wastewater and soil in cold countries. Acronyms: WW: Wastewater. NA: Not available

Country	Sampling period		Temperature (°C)		Sample		Reference
	Year	Months	Min	Max	Matrix	Type	
Austria	DS	March April- June -July	7.8	15.5	WW	Sewer	(Kretschmer et al., 2016)
China	2013 2014	January-April-June-October	15	30	WW	NA	(Liu et al., 2016)
Finland	2010 2011	Each season	9	18	WW	NA	(Karkman et al., 2016)
The Netherlands	2017 2018	December to May	9	15	WW	NA	(Barrios-Hernández et al., 2020)
The Netherlands	2009-2011		9	20	WW	NA	(Krzeminski et al., 2012)
Poland	2015	January-April-July-October	8.7	20.9	WW	Influent	(Osińska et al., 2017)
			9.5	21.3	WW	Effluent	
USA (Minnesota)	-	-	10	20	WW	Influent	(Johnston et al., 2019)
Czech Republic	1961-2000	Monthly	-0.5	21.2	Soil	-5 cm	(Pokladníková et al., 2008)
			0.4	19.9	Soil	-20 cm	
China (north)		Monthly average	-2	16	Soil		(Zhou et al., 2015)
Germany (Leibniz)	1894-2019	Monthly average	-7	35	Soil	-5 cm	1
			-9	27	Soil	-20 cm	
The Netherlands	2006-2020	Monthly average	1	21	Soil	-5cm	2
			1	21	Soil	-20 cm	

1 https://www.pik-potsdam.de/services/climate-weather-potsdam/climate-diagrams/ground-temperature

2 http://projects.knmi.nl/cabauw/insitu/observations/soiltemp/

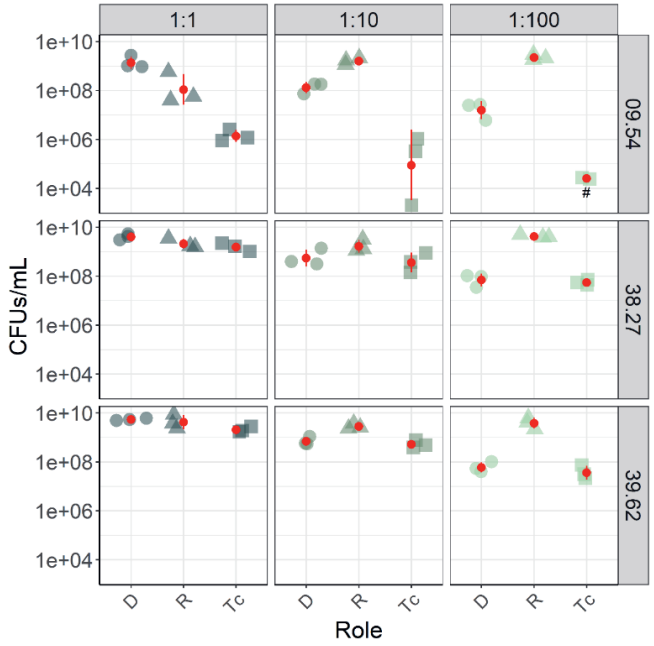


Figure S4.1. Absolute counts of donors (D), recipients (R), and transconjugants (Tc) after 2h-matings performed with different D/R proportions (1:1, 1:10, 1:100) for each strain. The averages and standard deviations of the matings are displayed in red

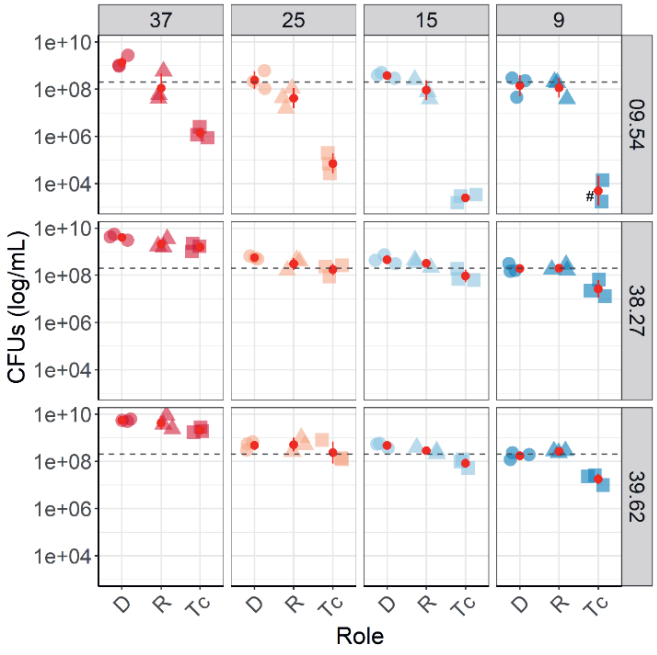


Figure S4.2. Absolute counts of donors (D), recipients (R), and transconjugants (Tc) after 2h-matings under diverse temperatures. The grey dashed line (2 x 10^8) indicates the approximate original number of cells in the beginning of the mating. "#" stands for the replicates with no detected transconjugants.

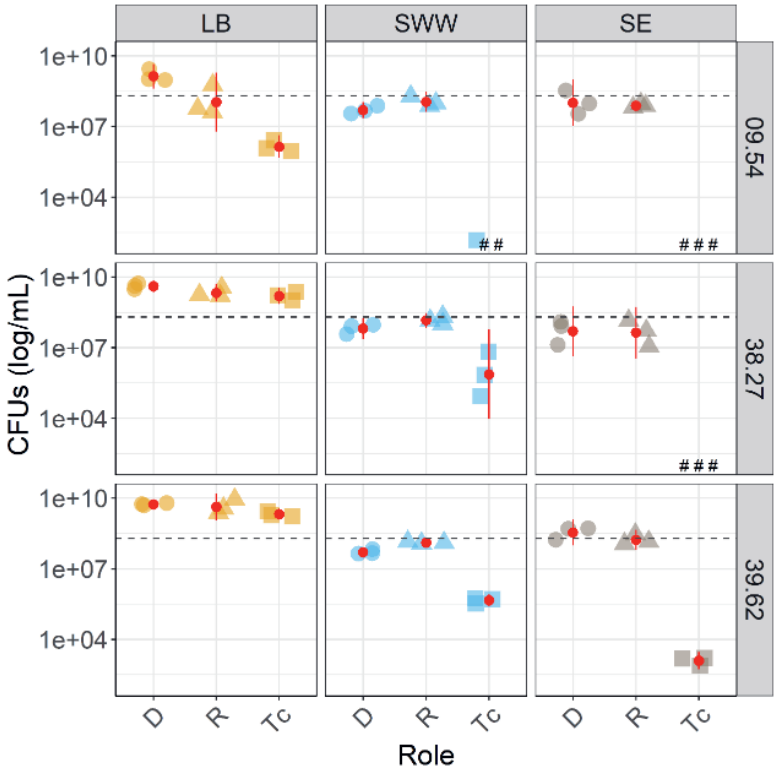


Figure S4.3. Absolute counts of donor (D), recipient (R) and transconjugants (Tc) after 2h mating in LB, Synthetic wastewater (SWW) and Soil Extract (SE) media. The discontinuous line at 2x10^8 indicates the approximate original number of cells in the beginning of the mating. "#" stands for the replicates with no detected transconjugants

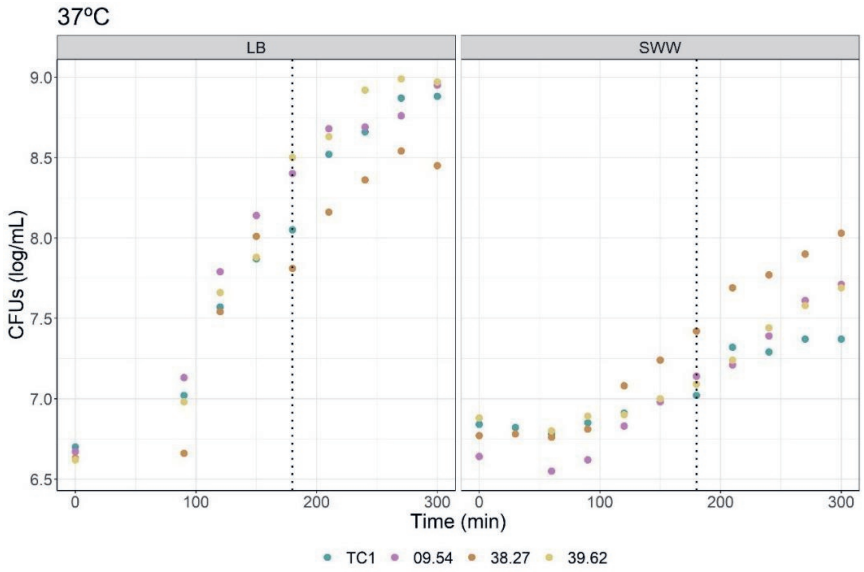


Figure S4.4. Growth curves in LB and synthetic wastewater (SWW) performed for the four tested strains, donor (TC1) and wild type recipients (09.54, 38.27 and 39.62). Vertical dashed line indicates 180 min (3h) of growth. No growth curve is presented for soil extract (SE) because no growth was observed in SE.

Figure S4:

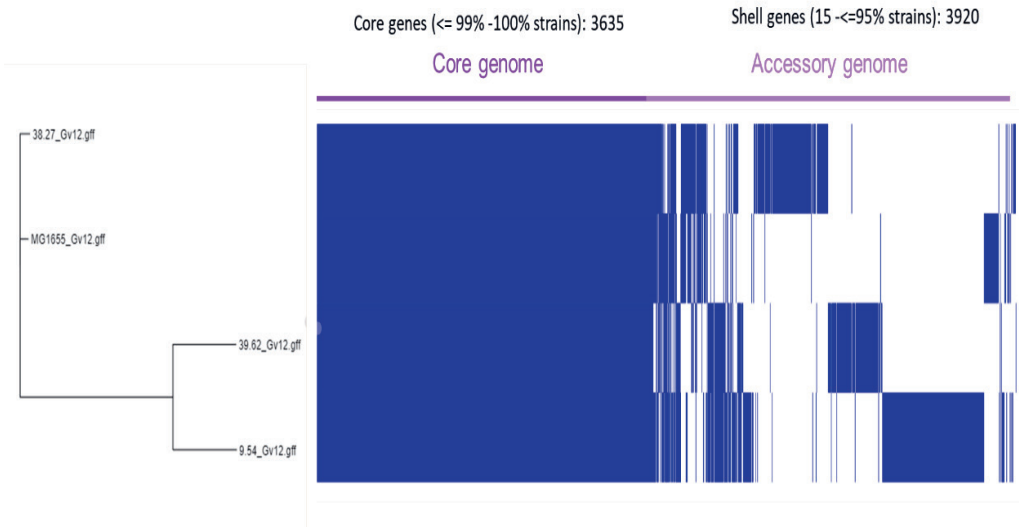


Figure S4.5. Core and accessory genome of the four strains used in this study. Since the sequence of the donor strain was not available, a RefSeq sequence of the same *E. coli* strain (MG1655, accession number NC_000913.3) was retrieved from GenBank for the analysis

4.8. References

Abis, K.L., Mara, D., 2006. Temperature measurement and stratification in facultative waste stabilisation ponds in the UK climate. Environ. Monit. Assess. 114, 35–47. https://doi.org/10.1007/s10661-006-1076-7

Al Qarni, H., Collier, P., O'Keeffe, J., Akunna, J., 2016. Investigating the removal of some pharmaceutical compounds in hospital wastewater treatment plants operating in Saudi Arabia. Environ. Sci. Pollut. Res. 23, 13003–13014. https://doi.org/10.1007/s11356-016-6389-7

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169. https://doi.org/10.1128/mr.59.1.143-169.1995

Bahl, M.I., Hansen, L.H., Sørensen, S.J., 2007. Impact of conjugal transfer on the stability of IncP-1 plasmid pKJK5 in bacterial populations. FEMS Microbiol. Lett. 266, 250–256. https://doi.org/10.1111/j.1574-6968.2006.00536.x

Bale, M.J., Fry, J.C., Day, M.J., 1988. Transfer and occurrence of large mercury resistance plasmids in river epilithon. Appl. Environ. Microbiol. 54, 972–978. https://doi.org/10.1128/aem.54.4.972-978.1988

Banerjee, G., Ray, A.K., Kumar, R., 2016. Effect of temperature on lateral gene transfer efficiency of multi-antibiotics resistant bacterium, alcaligenes faecalis. Sains Malaysiana 45, 909–914.

Barrios-Hernández, M.L., Pronk, M., Garcia, H., Boersma, A., Brdjanovic, D., van Loosdrecht, M.C.M., Hooijmans, C.M., 2020. Removal of bacterial and viral indicator organisms in full-scale aerobic granular sludge and conventional activated sludge systems. Water Res. X 6. https://doi.org/10.1016/j.wroa.2019.100040

Bellanger, X., Guilloteau, H., Bonot, S., Merlin, C., 2014a. Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review. Sci. Total Environ. 493, 872–882. https://doi.org/10.1016/j.scitotenv.2014.06.070

Bellanger, X., Guilloteau, H., Breuil, B., Merlin, C., 2014b. Natural microbial communities supporting the transfer of the IncP-1β plasmid pB10 exhibit a higher initial content of plasmids from the same incompatibility group. Front. Microbiol. 5, 1–8. https://doi.org/10.3389/fmicb.2014.00637

Benz, F., Huisman, J.S., Bakkeren, E., Herter, J.A., Stadler, T., Ackermann, M., Diard, M., Egli, A., Hall, A.R., Hardt, W.-D., Bonhoeffer, S., 2021. Plasmid- and strain-specific factors drive variation in ESBL-plasmid spread in vitro and in vivo. ISME J. 15, 862–878. https://doi.org/10.1038/s41396-020-00819-4

Bickel, S., Or, D., 2020. Soil bacterial diversity mediated by microscale aqueous-phase processes across biomes. Nat. Commun. 11, 116. https://doi.org/10.1038/s41467-019-13966-w

Binh, C.T.T., Heuer, H., Kaupenjohann, M., Smalla, K., 2008. Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS Microbiol. Ecol. 66, 25–37. https://doi.org/10.1111/j.1574-6941.2008.00526.x

Bradley, D.E., Taylor, D.E., Cohen, D.R., 1980. Specification of surface mating systems among conjugative drug resistance plasmids in Escherichia coli K-12. J. Bacteriol. 143, 1466–70. https://doi.org/10.1128/jb.143.3.1466-1470.1980

Carattoli, A., 2013. Plasmids and the spread of resistance. Int. J. Med. Microbiol. 303, 298–304. https://doi.org/10.1016/j.ijmm.2013.02.001

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., Møller Aarestrup, F., Hasman, H., 2014. In Silico Detection and Typing of Plasmids using PlasmidFinder and Plasmid Multilocus Sequence Typing. Antimicrob. Agents Chemother. 58, 3895–3903. https://doi.org/10.1128/AAC.02412-14

Ceccarelli, D., Kant, A., van Essen-Zandbergen, A., Dierikx, C., Hordijk, J., Wit, B., Mevius, D.J., Veldman, K.T., 2019. Diversity of Plasmids and Genes Encoding Resistance to Extended Spectrum Cephalosporins in Commensal Escherichia coli From Dutch Livestock in 2007–2017. Front. Microbiol. 10, 1–9. https://doi.org/10.3389/fmicb.2019.00076

Curtiss, R., Caro, L.G., Allison, D.P., Stallions, D.R., 1969. Early Stages of Conjugation in Escherichia coli. J. Bacteriol. 100, 1091–1104. https://doi.org/10.1128/jb.100.2.1091-1104.1969

Delatolla, R., Tufenkji, N., Comeau, Y., Gadbois, A., Lamarre, D., Berk, D., 2012. Effects of Long Exposure to Low Temperatures on Nitrifying Biofilm and Biomass in Wastewater Treatment. Water Environ. Res. 84, 328–338. https://doi.org/10.2175/106143012X13354606450924

Dimitriu, T., Marchant, L., Buckling, A., Raymond, B., 2019. Bacteria from natural populations transfer plasmids mostly towards their kin. Proc. R. Soc. B Biol. Sci. 286, 20191110. https://doi.org/10.1098/rspb.2019.1110

Fan, X.-T., Li, H., Chen, Q.-L., Zhang, Y.-S., Ye, J., Zhu, Y.-G., Su, J.-Q., 2019. Fate of Antibiotic Resistant Pseudomonas putida and Broad Host Range Plasmid in Natural Soil Microcosms. Front. Microbiol. 10, 1–10. https://doi.org/10.3389/fmicb.2019.00194

Fernandez-Astorga, A., Muela, A., Cisterna, R., Iriberri, J., Barcina, I., 1992. Biotic and abiotic factors affecting plasmid transfer in Escherichia coli strains. Appl. Environ. Microbiol. 58, 392–8. https://doi.org/10.1128/aem.58.1.392-398.1992

Fox, J., 2005. The R Commander: A Basic-Statistics Graphical User Interface to R. J. Stat. Softw. 14, 1–42. https://doi.org/10.18637/jss.v014.i09

Frank, J.A., Reich, C.I., Sharma, S., Weisbaum, J.S., Wilson, B.A., Olsen, G.J., 2008. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl. Environ. Microbiol. 74, 2461–2470. https://doi.org/10.1128/AEM.02272-07

Frost, L.S., Koraimann, G., 2010. Regulation of bacterial conjugation: balancing opportunity with adversity. Future Microbiol. 5, 1057–1071. https://doi.org/10.2217/fmb.10.70

Gama, J.A., Zilhão, R., Dionisio, F., 2017. Conjugation efficiency depends on intra and intercellular interactions between distinct plasmids: Plasmids promote the immigration of other plasmids but repress co-colonizing plasmids. Plasmid 93, 6–16. https://doi.org/10.1016/j.plasmid.2017.08.003

Goodman, A.E., Hild, E., Marshall, K.C., Hermansson, M., 1993. Conjugative plasmid transfer between bacteria under simulated marine oligotrophic conditions. Appl. Environ. Microbiol. 59, 1035–1040. https://doi.org/10.1128/aem.59.4.1035-1040.1993

Grabow, W.O.K., Prozesky, O.W., Burger, J.S., 1975. Behaviour in a river and dam of coliform bacteria with transferable or non-transferable drug resistance. Water Res. 9, 777–782. https://doi.org/10.1016/0043-1354(75)90134-7

Hadfield, J., Croucher, N.J., Goater, R.J., Abudahab, K., Aanensen, D.M., Harris, S.R., 2018. Phandango: an interactive viewer for bacterial population genomics. Bioinformatics 34, 292–293. https://doi.org/10.1093/bioinformatics/btx610

Headd, B., Bradford, S.A., 2020. The Conjugation Window in an Escherichia coli K-12 Strain with an IncFII Plasmid. Appl. Environ. Microbiol. 86, 1–18. https://doi.org/10.1128/AEM.00948-20

Headd, B., Bradford, S.A., 2018. Physicochemical factors that favor conjugation of an antibiotic resistant plasmid in non-growing bacterial cultures in the absence and presence of antibiotics. Front. Microbiol. 9, 1–14. https://doi.org/10.3389/fmicb.2018.02122

Hoang, V., Delatolla, R., Abujamel, T., Mottawea, W., Gadbois, A., Laflamme, E., Stintzi, A., 2014. Nitrifying moving bed biofilm reactor (MBBR) biofilm and biomass response to long term exposure to 1°C. Water Res. 49, 215–224. https://doi.org/10.1016/j.watres.2013.11.018

Hu, Y., Yang, X., Qin, J., Lu, N., Cheng, G., Wu, N., Pan, Y., Li, J., Zhu, L., Wang, X., Meng, Z., Zhao, F., Liu, D., Ma, J., Qin, N., Xiang, C., Xiao, Y., Li, L., Yang, H., Wang, Jian, Yang, R., Gao, G.F., Wang, Jun, Zhu, B., 2013. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. Nat. Commun. 4, 2151. https://doi.org/10.1038/ncomms3151

Inoue, D., Sei, K., Soda, S., Ike, M., Fujita, M., 2005. Potential of predominant activated sludge bacteria as recipients in conjugative plasmid transfer. J. Biosci. Bioeng. 100, 600–605. https://doi.org/10.1263/jbb.100.600

Jacquiod, S., Brejnrod, A., Morberg, S.M., Abu Al-Soud, W., Sørensen, S.J., Riber, L., 2017. Deciphering conjugative plasmid permissiveness in wastewater microbiomes. Mol. Ecol. 26, 3556–3571. https://doi.org/10.1111/mec.14138

Jaureguy, F., Landraud, L., Passet, V., Diancourt, L., Frapy, E., Guigon, G., Carbonnelle, E., Lortholary, O., Clermont, O., Denamur, E., Picard, B., Nassif, X., Brisse, S., 2008. Phylogenetic and genomic diversity of human bacteremic Escherichia coli strains. BMC Genomics 9, 560. https://doi.org/10.1186/1471-2164-9-560

Johnston, J., LaPara, T., Behrens, S., 2019. Composition and Dynamics of the Activated Sludge Microbiome during Seasonal Nitrification Failure. Sci. Rep. 9, 4565. https://doi.org/10.1038/s41598-019-40872-4

Karkman, A., Johnson, T.A., Lyra, C., Stedtfeld, R.D., Tamminen, M., Tiedje, J.M., Virta, M., 2016. High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. FEMS Microbiol. Ecol. 92, fiw014. https://doi.org/10.1093/femsec/fiw014

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945. https://doi.org/10.1038/ismej.2014.191

Kohyama, Y., Suzuki, S., 2019. Conjugative Gene Transfer between Nourished and Starved Cells of *Photobacterium damselae* ssp. *damselae* and *Escherichia coli*. Microbes Environ. 34, 388–392. https://doi.org/10.1264/jsme2.ME19099

Kretschmer, F., Simperler, L., Ertl, T., 2016. Analysing wastewater temperature development in a sewer system as a basis for the evaluation of wastewater heat recovery potentials. Energy Build. 128, 639–648. https://doi.org/10.1016/j.enbuild.2016.07.024

Krzeminski, P., Iglesias-Obelleiro, A., Madebo, G., Garrido, J.M., van der Graaf, J.H.J.M., van Lier, J.B., 2012. Impact of temperature on raw wastewater composition and activated sludge filterability in full-scale MBR systems for municipal sewage treatment. J. Memb. Sci. 423–424, 348–361. https://doi.org/10.1016/j.memsci.2012.08.032

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R.L., Jelsbak, L., Sicheritz-Pontén, T., Ussery, D.W., Aarestrup, F.M., Lund, O., 2012. Multilocus sequence typing of total-genome-sequenced bacteria. J. Clin. Microbiol. 50, 1355–1361. https://doi.org/10.1128/JCM.06094-11

Li, L., Dechesne, A., He, Z., Madsen, J.S., Nesme, J., Sørensen, S.J., Smets, B.F., 2018. Estimating the Transfer Range of Plasmids Encoding Antimicrobial Resistance in a Wastewater Treatment Plant Microbial Community. Environ. Sci. Technol. Lett. 5, 260–265. https://doi.org/10.1021/acs.estlett.8b00105

Li, L., Dechesne, A., Madsen, J.S., Nesme, J., Sørensen, S.J., Smets, B.F., 2020. Plasmids persist in a microbial community by providing fitness benefit to multiple phylotypes. ISME J. 14, 1170–1181. https://doi.org/10.1038/s41396-020-0596-4

Liu, T., Liu, S., Zheng, M., Chen, Q., Ni, J., 2016. Performance assessment of full-scale wastewater treatment plants based on seasonal variability of microbial communities via high-throughput sequencing. PLoS One 11. https://doi.org/10.1371/journal.pone.0152998

MacDonald, J.A., Smets, B.F., Rittmann, B.E., 1992. The effects of energy availability on the conjugative-transfer kinetics of plasmid RP4. Water Res. 26, 461–468. https://doi.org/10.1016/0043-1354(92)90046-7

Manti, A., Boi, P., Falcioni, T., Canonico, B., Ventura, A., Sisti, D., Pianetti, A., Balsamo, M., Papa, S., 2008. Bacterial Cell Monitoring in Wastewater Treatment Plants by Flow Cytometry. Water Environ. Res. 80, 346–354. https://doi.org/10.2175/106143007x221418

Musovic, S., Dechesne, A., Sørensen, J., Smets, B.F., 2010. Novel Assay To Assess Permissiveness of a Soil Microbial Community toward Receipt of Mobile Genetic Elements. Appl. Environ. Microbiol. 76, 4813–4818. https://doi.org/10.1128/AEM.02713-09

Musovic, S., Oregaard, G., Kroer, N., Sørensen, S.J., 2006. Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among gram-positive and gram-negative bacteria indigenous to the barley rhizosphere. Appl. Environ. Microbiol. 72, 6687–6692. https://doi.org/10.1128/AEM.00013-06

Norman, A., Riber, L., Luo, W., Li, L.L., Hansen, L.H., Sørensen, S.J., 2014. An Improved Method for Including Upper Size Range Plasmids in Metamobilomes. PLoS One 9, e104405. https://doi.org/10.1371/journal.pone.0104405

Novick, R.P., 1987. Plasmid incompatibility. Microbiol. Rev. 51, 381–95. https://doi.org/10.1128/mr.51.4.381-395.1987

O'Morchoe, S.B., Ogunseitan, O., Sayler, G.S., Miller, R. V., 1988. Conjugal transfer of R68.45 and FP5 between Pseudomonas aeruginosa strains in a freshwater environment. Appl. Environ. Microbiol. 54, 1923–1929. https://doi.org/10.1128/AEM.54.8.1923-1929.1988

Ochman, H., Lawrence, J.G., Groisman, E.A., 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405, 299–304. https://doi.org/10.1038/35012500

Osińska, A., Korzeniewska, E., Harnisz, M., Felis, E., Bajkacz, S., Jachimowicz, P., Niestępski, S., Konopka, I., 2020. Small-scale wastewater treatment plants as a source of the dissemination of antibiotic resistance genes in the aquatic environment. J. Hazard. Mater. 381, 121221. https://doi.org/10.1016/j.jhazmat.2019.121221

Osińska, A., Korzeniewska, E., Harnisz, M., Niestępski, S., 2017. The prevalence and characterization of antibiotic-resistant and virulent Escherichia coli strains in the municipal wastewater system and their environmental fate. Sci. Total Environ. 577, 367–375. https://doi.org/10.1016/j.scitotenv.2016.10.203

Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T.G., Fookes, M., Falush, D., Keane, J.A., Parkhill, J., 2015. Roary: Rapid large-scale prokaryote pan genome analysis. Bioinformatics 31, 3691–3693. https://doi.org/10.1093/bioinformatics/btv421

Pallares-Vega, R., Hernandez Leal, L., Fletcher, B.N., Vias-Torres, E., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2021. Annual dynamics of antimicrobials and resistance determinants in flocculent and aerobic granular sludge treatment systems. Water Res. 190, 116752. https://doi.org/10.1016/j.watres.2020.116752

Pinilla-Redondo, R., Cyriaque, V., Jacquiod, S., Sørensen, S.J., Riber, L., 2018. Monitoring plasmid-mediated horizontal gene transfer in microbiomes: recent advances and future perspectives. Plasmid 99, 56–67. https://doi.org/10.1016/j.plasmid.2018.08.002

Pokladníková, H., Rožnovský, J., Středa, T., 2008. Evaluation of soil temperatures at agroclimatological station pohořelice. Soil Water Res. 3, 223–230. https://doi.org/10.17221/2092-swr

Popowska, M., Krawczyk-Balska, A., 2013. Broad-host-range IncP-1 plasmids and their resistance potential. Front. Microbiol. 4, 1–8. https://doi.org/10.3389/fmicb.2013.00044

Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2 - Approximately maximum-likelihood trees for large alignments. PLoS One 5. https://doi.org/10.1371/journal.pone.0009490

R Core Team, 2018. R: A language and environment for statistical computing. R Found. Stat. Comput. Vienna.

Rochelle, P.A., Fry, J.C., Day, M.J., 1989. Factors affecting conjugal transfer of plasmids encoding mercury resistance from pure cultures and mixed natural suspensions of epilithic bacteria. J. Gen. Microbiol. 135, 409–424. https://doi.org/10.1099/00221287-135-2-409

Rodrigues, D.F., Tiedje, J.M., 2008. Coping with our cold planet. Appl. Environ. Microbiol. 74, 1677–1686. https://doi.org/10.1128/AEM.02000-07

Rottiers, A., Boeije, G., 1999. Part II: Design and validation of test units. Science (80-.). 38, 711–727.

Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Guerra, B., Mevius, D.J., Hordijk, J., 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J. Antimicrob. Chemother. 73, 1121–1137. https://doi.org/10.1093/jac/dkx488

Rozwandowicz, M., Hordijk, J., Bossers, A., Zomer, A.L., Wagenaar, J.A., Mevius, D.J., Brouwer, M.S.M., 2020. Incompatibility and phylogenetic relationship of I-complex plasmids. Plasmid 109, 102502. https://doi.org/10.1016/j.plasmid.2020.102502

Sakuda, A., Suzuki-Minakuchi, C., Matsui, K., Takahashi, Y., Okada, K., Yamane, H., Shintani, M., Nojiri, H., 2018. Divalent cations increase the conjugation efficiency of the incompatibility P-7 group plasmid pCAR1 among different pseudomonas hosts. Microbiol. (United Kingdom) 164, 20–27. https://doi.org/10.1099/mic.0.000583

Schmitt, H., Blaak, H., Hoeksma, P., van de Schans, M., Kemper, M., de Rijk, S., de RodaHusman, A., 2019. Antibioticaresistente bacteriën, resistentiegenen en antibioticaresiduen in mest, RIMV. https://doi.org/10.21945/RIVM-2019-0112

Scott, K.P., Mercer, D.K., Glover, L.A., Flint, H.J., 2006. The green fluorescent protein as a visible marker for lactic acid bacteria in complex ecosystems. FEMS Microbiol. Ecol. 26, 219–230. https://doi.org/10.1111/j.1574-6941.1998.tb00507.x

Seemann, T., 2014. Prokka: Rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069. https://doi.org/10.1093/bioinformatics/btu153

Sengeløv, G., Kristensen, K.J., Sørensen, A.H., Kroer, N., Sørensen, S.J., 2001. Effect of Genomic Location on Horizontal Transfer of a Recombinant Gene Cassette Between Pseudomonas Strains in the Rhizosphere and Spermosphere of Barley Seedlings. Curr. Microbiol. 42, 160–167. https://doi.org/10.1007/s002840010197

Seoane, J., Yankelevich, T., Dechesne, A., Merkey, B., Sternberg, C., Smets, B.F., 2011. An individual-based approach to explain plasmid invasion in bacterial populations. FEMS Microbiol. Ecol. 75, 17–27. https://doi.org/10.1111/j.1574-6941.2010.00994.x

Shintani, M., Nour, E., Elsayed, T., Blau, K., Wall, I., Jechalke, S., Spröer, C., Bunk, B., Overmann, J., Smalla, K., 2020. Plant Species-Dependent Increased Abundance and Diversity of IncP-1 Plasmids in the Rhizosphere: New Insights Into Their Role and Ecology. Front. Microbiol. 11, 1–11. https://doi.org/10.3389/fmicb.2020.590776

Smillie, C., Garcillán-Barcia, M.P., Francia, M.V., Rocha, E.P.C., de la Cruz, F., 2010. Mobility of plasmids. Microbiol. Mol. Biol. Rev. 74, 434–52. https://doi.org/10.1128/MMBR.00020-10

Smith, E.M., Prairie, Y.T., 2004. Bacterial metabolism and growth efficiency in lakes: The importance of phosphorus availability. Limnol. Oceanogr. 49, 137–147. https://doi.org/10.4319/lo.2004.49.1.0137

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., 2005. Studying plasmid horizontal transfer in situ: a critical review. Nat. Rev. Microbiol. 3, 700–710. https://doi.org/10.1038/nrmicro1232

Thomas, C.M., Smith, C.A., 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. 41, 77–101. https://doi.org/10.1146/annurev.mi.41.100187.000453

Trevors, J.T., Bej, A.K., Mojib, N., van Elsas, J.D., van Overbeek, L., 2012. Bacterial gene expression at low temperatures. Extremophiles 16, 167–176. https://doi.org/10.1007/s00792-011-0423-y

Vidgren, V., Gibson, B., 2018. Trans-regulation and localization of orthologous maltose transporters in the interspecies lager yeast hybrid. FEMS Yeast Res. 18, 1–14. https://doi.org/10.1093/femsyr/foy065

Vinet, L., Zhedanov, A., 2010. A “missing” family of classical orthogonal polynomials. https://doi.org/10.1088/1751-8113/44/8/085201

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C.J., Ochman, H., Achtman, M., 2006. Sex and virulence in Escherichia coli: An evolutionary perspective. Mol. Microbiol. 60, 1136–1151. https://doi.org/10.1111/j.1365-2958.2006.05172.x

Zhong, X., Kro ̈l, J.E., Top, E.M., Krone, S.M., 2010. Accounting for mating pair formation in plasmid population dynamics. J. Theor. Biol. 262, 711–719. https://doi.org/10.1016/j.jtbi.2009.10.013

Zhou, Z., Xu, M., Kang, F., Sun, O.J., 2015. Maximum temperature accounts for annual soil CO2 efflux in temperate forests of Northern China. Sci. Rep. 5, 1–10. https://doi.org/10.1038/srep12142

CHAPTER 5

5

Potential transfer *in situ* of broad and narrow host range plasmids under different redox conditions and temperatures

Abstract

Horizontal gene transfer through conjugal plasmids is key in disseminating antibiotic-resistant determinants in engineered environments such as activated sludge. However, monitoring plasmid transfer in complex environmental and engineered matrices remains challenging. The use of fluorescently tagged plasmids and donor cells combined with flow cytometry and cell sorting constitutes a valuable tool to study plasmid transfer and community permissiveness, yet their use remains coupled to culture-based mating. Here, we used a fluorescently labelled donor and plasmids coupled with direct measurement of transconjugants by flow cytometry. The transfer was also assessed by culturing methods. By those means, we evaluated *in situ* the conjugal transfer of a broad (IncP-1) and a narrow (IncI) host range plasmid towards indigenous activated sludge microbiota under different temperatures (30°C and 15°C) and redox conditions (aerobic vs anaerobic). Conjugal transfer was only observed for the IncP-1 plasmid (10^{-4} to 10^{-6} T/D), and its detection by flow cytometry was favored under aerobic conditions and mesophilic temperature (30°C). Donor persistence under environmental psychrophilic temperatures (15°C) or anaerobic conditions hindered the quantification by flow cytometry. Moreover, the low number of recovered transconjugants prevented the direct evaluation of their identity.

.

Rebeca Pallares-Vega, Asmus Kalckar Olesen, Lucia Hernandez Leal, Mark. C.M. van Loosdrecht, David G. Weissbrodt, Søren Sørensen, and Heike Schmitt

5.1. Introduction

Conjugal plasmids can be exchanged promiscuously between diverse bacteria and bacterial species contributing to bacterial genome plasticity (Sørensen et al., 2005). A considerable proportion of conjugal plasmids contains antibiotic resistance genes (ARGs) among other beneficial traits for their bacterial host (i.e. virulence factors or secondary metabolism enzymes) (Rankin et al., 2011). The exchange of antibiotic resistance plasmids fosters the dissemination of ARGs across unrelated taxa and the development of novel multi-resistant bacteria (Carattoli, 2013). Thus, the conjugal transfer of resistant plasmids represents a challenge in the fight against antibiotic resistance, a significant threat to human health.

Gut bacteria, from both humans and livestock, comprise a reservoir of resistance plasmids. (Francino, 2016). Gut microorganisms, including antibiotic resistant bacteria and their resistance determinants, are continuously discharged into the environment together with the feces. In high-income countries, fecal material along with fecal microbiota, are collected by the sewage system and treated in wastewater treatment plants (WWTPs) (Manaia et al., 2018). A substantial proportion of the gut microbiota might not survive long under the conditions of the sewer or the WWTP (Cai et al., 2014), for instance due to evolving redox conditions (Quintela-Baluja et al., 2019). However, they may be able to horizontally transfer their traits to the indigenous bacteria, better suited to endure in the receiving environment. These indigenous bacteria might thrive through the wastewater treatment (or even be part of it), with a potential to reach freshwater ecosystems. Thus, conjugal transfer of resistant plasmids might enlarge and shape the environmental resistome (Perry and Wright, 2013). Ultimately, resistant plasmids might be further transferred to environmental bacteria that can be potentially infectious agents such as *Pseudomonas* spp. or *Acinetobacter* spp.

Conjugation is a process that requires cell proximity and therefore, is favoured in environments with high cell densities (Seoane et al., 2011). Within wastewater treatment, activated sludge in the biological system represents a high cell density hotspot for the exchange and spread of plasmid-mediated ARGs. Selection of transconjugants might be favoured by selectors such as heavy metals or antibiotics, commonly found in activated sludge settings (Baker-Austin et al., 2006; Berendonk et al., 2015). Despite their importance, the dynamics of plasmid transfer in activated sludge are still insufficiently understood mostly due to the constraints of monitoring conjugation *in situ* (Pinilla-Redondo et al., 2018; Sørensen et al., 2005). Only a few studies have successfully assessed resistance plasmid transfer in bioreactors

or microcosms simulating activated sludge conditions (Bellanger et al., 2014a; Bonot and Merlin, 2010; Mantilla-Calderon and Hong, 2017; Merlin et al., 2011; D. Yang et al., 2013). However, these works provide no or limited information on the identity of the recipients. The development of fluorescently labelled strains and their combination with flow cytometry and cell sorting has encompassed a promising option to unravel the identity of the recipients (community permissiveness). Although initially designed for *in situ* application (Geisenberger et al., 1999; Seoane et al., 2011), the recent combination of these techniques with classical filter mating have allowed evaluating the community permissiveness of complex microbial communities such as soil (Klümper et al., 2015), wastewater (Jacquiod et al., 2017), and activated sludge (Li et al., 2020; Li et al., 2018a). This experimental approach is still biased towards selecting the small (1%) proportion of culturable bacteria. Moreover, since they aim to investigate the maximum number of potential recipients, favourable conditions (i.e., warm temperature or abundant nutrients) are applied. Thus, the aforementioned settings are not fully representative of real conditions in wastewater environments and might be overestimating the real transfer rate under *in situ* conditions.

Generally, broad host range plasmids, mainly from the IncP-1 and IncQ families (Bellanger et al., 2014; Jacquiod et al., 2017; Klümper et al., 2015; L. Li et al., 2020; Li et al., 2018a) are the chosen vectors used in the majority of conjugation studies. IncP-1 plasmids are notoriously abundant in aquatic environments and activated sludge systems (Pallares-Vega et al., 2021a) which may point to their key role in those environments and their potential for genetic exchanges within the WWTPs ecosystem. However, enteric bacteria such as those in the Enterobacteriaceae family (*E. coli*, *Klebsiella* spp., *Salmonella* spp.), contain other types of plasmids potentially harbouring the most relevant and novel ARGs from the clinical perspective (i.e., extended-spectrum beta-lactams or carbapenems). These plasmids generally belong to the IncF, IncI and IncK families (Rozwandowicz et al., 2018). The potential exchange of these plasmids in complex activated sludge systems is presumably low because of their narrow host range (thought to be limited to Enterobacteriaceae family), albeit no comparison has been made yet. Up to date, the investigation of narrow host range plasmids in activated sludge microcosms is anecdotic (Mantilla-Calderon and Hong, 2017).

Differences in sludge community within the activated sludge, for instance, following the redox phases within the treatment, might also affect the transfer rate. Previous works have observed a higher plasmid transfer in aerobic conditions when compared to anoxic (Merlin et

al., 2011) or anaerobic (Mantilla-Calderon and Hong, 2017). Likely, the recipients' identity will also vary, but this has not been explored yet in culture-independent experiments.

The main scope of this work was to quantify the differences in conjugation dynamics between broad (IncP-1) and narrow (IncI) host range plasmids within activated sludge microcosms. Moreover, we investigated the role of key conditions such as redox (aerobic versus anoxic) and also temperature (30°C vs 15°C).

5.2. Material and Methods

5.2.1. Bacteria strains, plasmids and recipient community.

E. coli K12 MG1665::lacIq-mCherry-Kan^R with either broad host range IncP-1 plasmid pKJK5 (54kbp) km^R, Tmp^R , Smx^R or narrow host range IncI plasmid R64 (121 kbp), Str^R, Kan^R were used. The donor strain (*E. coli* MG1665) and the plasmid pKJK5 (IncP-1) have been previously described in (Klümper et al., 2014). The complete sequence of the IncI plasmid R64 has also been defined elsewhere (Sampei et al., 2010). The donor strain is chromosomally tagged with the red fluorescent label mCherry and a constitutively expressed laqI^q repressor. Both plasmids are also tagged with the green fluorescence protein (gfpmut3), placed downstream a lac promoter which is repressed by lacI^q in the donor cells.

5.2.2. Culture media.

Donors were routinely cultured in Luria Bertani (LB) Broth at 37°C supplemented with kanamycin 50 µg mL^{-1} (Kan) and either trimethoprim (Tmp) 32 µg mL^{-1} for IncP-1 or streptomycin (Str) 100 µg mL^{-1} for IncI. Transconjugants were recovered in LB agar plates supplemented with Kan 100 µg mL^{-1}, and either Tmp 32 µg mL^{-1} and sulfamethoxazole 128 µg mL^{-1} for experiments with pKJK5 or Str 100 µg mL^{-1} for experiments with R64. Agar plates were also complemented with nystatin 50 µg mL^{-1} to avoid fungal growth.

Synthetic Wastewater (SWW) was prepared as previously mentioned in (Pallares-Vega et al., 2021b). This medium was designed to represent the average nutrient concentration of Dutch wastewater (approximately 500 mg COD L^{-1}, 50 mg L^{-1} Nitrogen and 7 mg L^{-1} Phosphorus 1.4 mg L^{-1}). SWW was supplemented with uridine at a final concentration of 60 µg mL^{-1} because the donor strain has a mutation in the *rph-pyrE* operon and suffers from pyrimidine starvation in minimal media (Jensen, 1993) such as the SWW.

5.2.3. Activated sludge sampling and preparation and microcosms preparation.

Sludge samples were obtained from the municipal wastewater treatment plant (WWTP)

(Mølleåværket, Lyngby-Taarbæk, Denmark) at the beginning of each week. Grab samples were taken from the collector of the aeration basin and stored in a refrigerated container until being processed in the laboratory. Samples were subjected to analysis of total solids (TS) and volatile solids analysis (VS) following standard procedures (Clesceri et al., 1998). Samples were stored at 4°C upon use.

For aerobic microcosms, Erlenmeyer flasks of 350 mL volume were used to prepare the microcosms. A total volume of sludge of 80 mL (or equivalent to achieve a VS content of ca. 2g kg^{-1}) was used. The sludge was amended with 10 mL of a 10-fold concentrated solution of Synthetic Wastewater (SWW) as aforementioned. This nutrient amendment sums up to ca. 500 mg COD mg L^{-1} day^{-1}, which corresponds to an approximate organic loading rate of 0.16 kg COD $kgTS^{-1}day^{-1}$.

Overnight donor cultures were centrifuged at 10.000 x g and washed twice in saline solution (NaCl 0.89% w/v) before being resuspended in 10 mL of saline solution, at an approximate concentration of ca $2\cdot 10^9$ CFUs mL^{-1} and added to the culture flasks. The expected initial concentration of donors was, therefore, ca. $2\cdot 10^8$ CFUs mL^{-1}. Cultures were incubated at 30 or 15°C in an orbital shaker for 24h at 250 rpm. Six biological replicates were performed per experiment, divided in two different experimental days. A blank microcosm for each temperature (30 or 15°C) was prepared for each experimental day. The blank microcosms contained the same components as the sample microcosms, with the exception of the donor inoculum, which was substituted by 10 mL of sterile saline solution (NaCl 0.89% w/v).

For anaerobic microcosms, 250 mL serum bottles with isopropyl rubber stoppers were used. Activated sludge was pre-conditioned prior to the anaerobic phase (i.e., allowing for intracellular phosphorus accumulation) by bubbling air for 30 min at room temperature. Then anaerobic microcosms were prepared in a total volume of 50 mL, containing 37.5 mL of sludge (at a final VS concentration of 2 g VS Kg^{-1} of activated sludge) and 3.5 mL of 10-fold concentrated synthetic wastewater to reach a final concentration of 500 mg COD L^{-1} to maintain the organic loading rate as aforementioned for the aerobic experiments.

Microcosms were subsequently spiked with 5 mL of donor cells (to a final concentration of $2\cdot 10^8$ CFUs mL^{-1}) prepared as indicated above and completed up to 50 mL with sterile water. Anaerobic conditions were achieved as follow: serum flasks were tightly closed and flushed with N_2 gas for 10 min in the liquid phase and 10 min for the headspace while maintained on ice. Flasks were then incubated at 30 or 15°C at 150 rpm. Before and after incubation, gas samples were taken from the headspace to confirm the lack of oxygen in the headspace and stored in 6mL exetainer vacuum vials upon analysis. The presence of N_2, O_2 and CO_2 was analysed by

gas chromatography (CP4900 Micro GC, Varian, NL). Further description of the device can be found in Kiragosyan et al. (2020).

5.2.4. Cell recovery

After incubation, the conjugation rate and transconjugants were evaluated by culture-dependent (CFU enumeration by plate counting) and culture-independent methods (flow cytometry). A workflow scheme is provided in **Figure 5.1.**

For culture-dependent analyses, 1 mL of each microcosm was serially diluted and plated in LB agar plates supplemented with antibiotics and nystatin as above mentioned. Plates were then incubated at 30°C for 24h, following by CFU enumeration of donors and transconjugants. Transconjugants were differentiated from donors by their expression of GFP protein. Observation of the fluorescence was evaluated with the help of a Dark Reader transilluminator with an amber screen (Clare Chemical Research, US). Serial dilutions of raw activated sludge were also incubated in the same media to assess the background level of resistance to the corresponding antibiotics.

For the culture-independent evaluation, 40 mL of the microcosm were transferred to falcon tube, vigorously vortexed and sonicated in an ultrasonic cleaner (USC1200 THD, VWR, DK) for 5 minutes at 25°C. Afterwards, cells were recovered with a Nycodenz density gradient method: 1.2 mL of homogenized microcosm were added on top of 700 µL of Nycodenz solution (50% w/v, Progen, FR) in a 2mL microcentrifuge tube. A total of five tubes were prepared from each microcosm. The microcentrifuge tubes were centrifuged at 10.000 xg at 4°C for 25 min. After centrifugation, the phase on top of the Nycondenz layer was resuspended into 5 mL of pyrophosphate buffer (50 mM $Na_4O_7P_2$). All five tubes from each microcosm replicate were resuspended in the same tube. Tubes were centrifuged for 10 min at 10.000 x g at 4°C, and the supernatant was discarded. The remaining pellet was resuspended in 1 mL 0.9% of NaCl solution, and filtered through a 10 µm pore size nylon membrane filter (Frisnette, DK). Filtrates were kept overnight at 4°C for the maturation of the fluorophores and then resuspended in 25% glycerol and stored at -80°C upon analysis by flow cytometry.

5.2.5. Flow cytometry and cell sorting

For the detection of total cells, vials were thawed on ice and subsequently centrifuged at 10.000 x g for 5 min at 4 °C and resuspended in 1mL of PBS. Cells were then evaluated by means of flow cytometry with FACSAria IIIu (Becton Dickson Biosciences, US). Samples were prepared by mixing 100 µL of recovered cells with 1 mL of PBS. When the number of events

was low, cells and PBS ratio were adapted to reach a range of 1000 to 3000 events s^{-1}. The detection of donors and transconjugants was based on their expression of mCherry and GFP proteins, respectively. The mCherry fluorophore was excited by a 561 nm laser and detected in the PE Texas Red channel, while GFP protein was excited by a 488nm laser and detected in the FITC. A complete description of the technical details can be found in Pinilla-Redondo et al. (2020) with the modification that the targeted events were increased to 10^6 instead of 10^5.

Because of the low number of detected transconjugants, cell sorting was only performed for the IncP-1 aerobic experiments by sorting all the transconjugants cells from the six replicates into a single tube. A total of 500 transconjugant cells were sorted into a 5 mL sterile polypropylene round-bottom tube (Falcon by Corning, US) containing 0.5 mL of PBS. DNA extraction and subsequent 16S rRNA subunit sequencing was assumed not to be possible on such a low amount of transconjugants. A culture-based approach was chosen instead. Recovered cells were resuspended first in SWW media and incubated in an orbital shaker at 30°C and 200 rpm for 48h. As no growth was observed after 48h, an amendment with Tryptic Soy Agar was performed. Cells were again incubated for 24h at 30 °C, after which growth was observed, unfortunately corresponding to donor contamination as observed by flow cytometry.

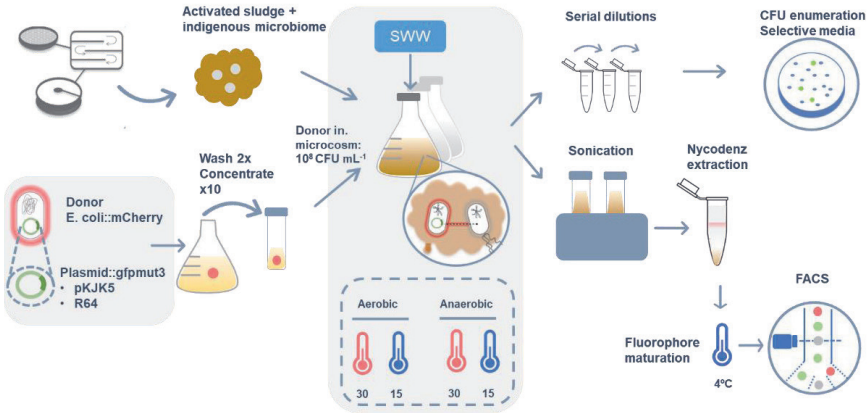


Figure 5.1. Workflow of microcosms set up and downstream analysis by flow cytometry (FACS) and CFU enumeration on selective media. Different experimental combinations are highlighted with a grey background.

5.2.6. Data analysis:

For the comparison of donor or transconjugants across different temperatures or redox conditions (measured by either flow cytometry or plate counting), outcome variables (log-transformed flow cytometry events or CFU mL-1 counts) grouped by temperature and redox were tested for normality with the Shapiro-Wilk test. When verified, a t-test was performed.

When normality was not achieved, the non-parametric version, a two sample Wilcoxon test, was used instead. In both cases, the significance level was established at 0.05. All analysis were performed in R version 3.6.1 (R Core Team, 2018) and Rstudio (http://www.rstudio.com/) version 1.2.5001.

5.3. Results.

5.3.1 Limited transfer of IncP-1 and non-detectable from IncI plasmid.

Conjugal transferability of pKJK5 (IncP-1) and R64 (IncI) from *E. coli* towards the indigenous activated sludge microbiota at mesophilic (30°C) and environmental relevant temperatures (psychrophilic, 15°C) was assessed in sludge microcosms.

A significantly higher number of donors was observed in the assays performed at 15°C in comparison to 30°C (+1.5 logs, $p<0.01$) (**Figure 5.2**). Transconjugants carrying the broad host range plasmid pKJK5 were detected in the two tested temperatures (30 and 15°C). The transfer rate expressed as transconjugants to donor ratio (T/D) at 30°C was $2.2 \cdot 10^{-3}$ according to flow cytometry and $1.9 \cdot 10^{-4}$ measured by CFU enumeration (**Figure S5.1**). At 15°C, the transfer rate (T/D) was on average $8.7 \cdot 10^{-6}$ measured by flow cytometry and $1.3 \cdot 10^{-6}$ by CFU enumeration. Detectable transfer of R64 could be observed neither by culturing methods and by flow cytometry (only two replicates presented single events counted as transconjugants, but those were below the quantification limit of 3 events in 10^6 events set to discard possible background signal).

Results for donor counts by flow cytometry were consistent with those of culture-based analysis. Contrarily, a significantly higher number of transconjugants (ca. 1 log, $p<0.05$) was detected at 30°C in comparison with 15°C by flow cytometry but not by culture-based methods.

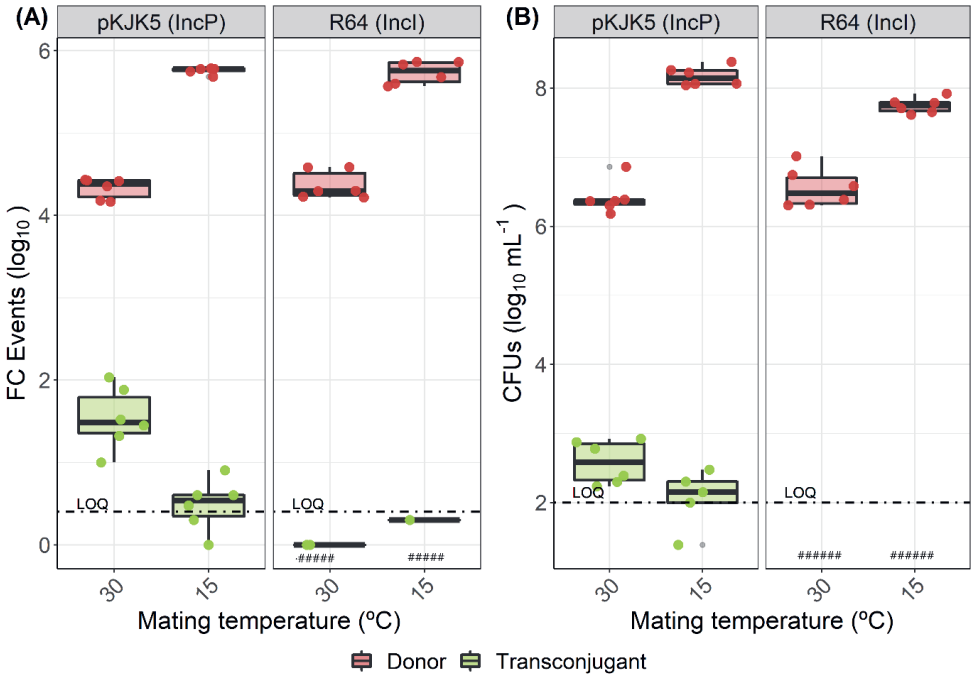


Figure 5.2. Donor persistence and transconjugants of pKJK5 (IncP-1) and R64 (IncI) plasmids in sludge microcosms under different temperatures. Enumeration of cells was performed by flow cytometry (A) and plate count (B). Plasmid transfer was only observed for pKJK5 and was favoured at mesophilic temperatures (30°C). LOQ represents the limit of quantification of each technique. "#" stands for each replicate measured without a detectable transconjugant. Donor was spiked at an initial concentration of circa 2 ·10^8 CFUs mL^{-1}

5.3.2. Transfer of IncP-1 plasmids under aerobic vs anaerobic conditions.

Conjugal transferability of pKJK5 to indigenous activated sludge under anaerobic conditions and different temperatures was assessed in sludge microcosms and compared to the previously described aerobic experiments.

Under anaerobic conditions, transconjugants were barely detected by flow cytometry: only a few replicates yielded transconjugants above the limit of quantification (3 events per 10^6 events). Contrarily, transconjugants were observed in the same range as in the aerobic conditions by CFU enumeration (**Figure 5.3**).

In comparison with aerobic conditions, the number of detectable donors after anaerobic mating at 30°C was moderately higher (0.3 – 1 log) than in aerobic conditions. This was observed by both flow cytometry and CFU enumeration. After mating at 15°C, donor numbers were only slightly lower (-0.2 logs) in anaerobic assays than in aerobic assays.

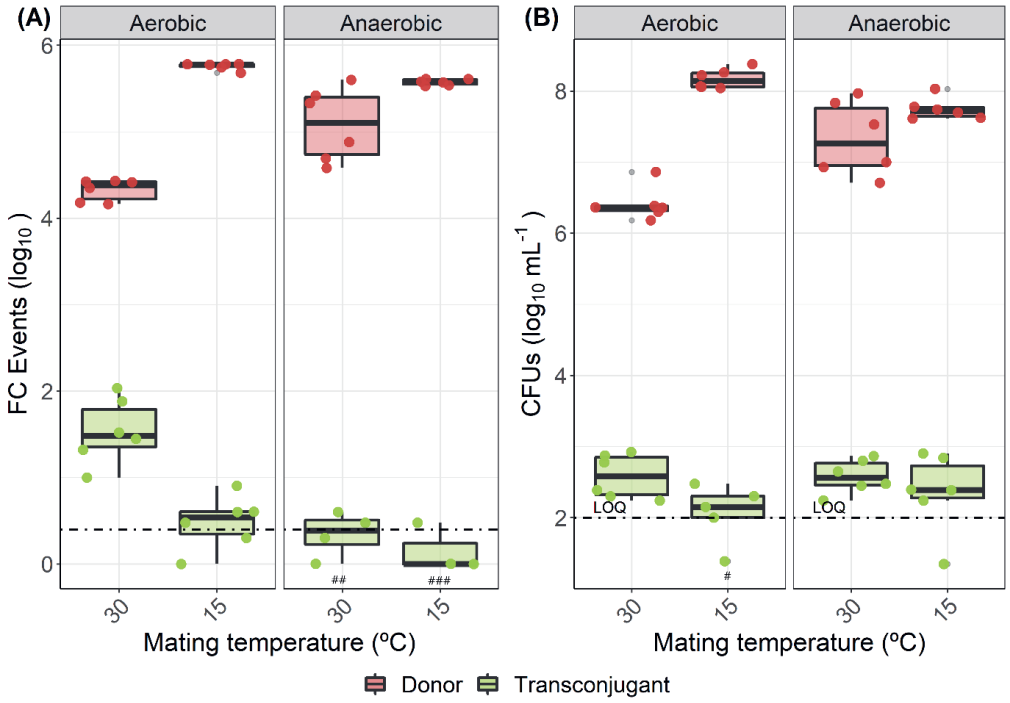


Figure 5.3. Donor persistence and conjugal transfer of IncP-1 pKJK5 under in sludge microcosms different redox conditions, measured by flow cytometry (A) and plate count (B). Plasmid transfer was possible in both redox conditions but was favoured under aerobic conditions at mesophilic temperatures. LOQ represents the limit of quantification for each technique. "#" stands for each replicate measured without a detectable transconjugant. Donor was spiked at an initial concentration of circa $2 \cdot 10^8$ CFUs mL^{-1}

5.4. Discussion

Antimicrobial resistance dissemination through horizontal gene transfer is a multidimensional problem. To prioritize mitigation efforts, it is fundamental to identify which key elements contribute to this phenomenon. In the context of complex environmental and wastewater engineering matrices, narrow host range plasmids might not be as relevant as broad host range plasmids because their dissemination capacity is restricted to a few species of the family Enterobacteriaceae (Rozwandowicz et al., 2018), not predominant in the sludge. However, this hypothesis needs to be assessed with quantitative analysis. We used culture-dependent and independent methods to measure the conjugal transfer of plasmids from different host ranges towards indigenous activated sludge bacteria in microcosm. The mating conditions aimed to evaluate relevant abiotic settings for activated sludge systems such as temperature and oxygen presence.

5.4.1. Successful detection of transfer events in situ for pKJK5 but not for R64.

Under aerobic conditions, the rate of conjugal transfer could only be quantified for the broad host range plasmid pKJK5 (IncP-1). In assays with the narrow host range plasmid R64 (IncI) only single events were detected as transconjugants by flow cytometry (below the limit of quantification, thus considered noise), and no transconjugants were detected by CFU enumeration.

The successful transfer of pKJK5 was expected, as this plasmid is highly promiscuous, and many bacterial genera within the activated sludge and wastewater community support its transfer (Jacquiod et al., 2017; Li et al., 2020). Here, community permissiveness could not be assessed. Although transconjugants harbouring pKJK5 were detected in all replicates, the number of sorted cells by FACS (of a shared pool of the six replicates) was insufficient to perform direct DNA extraction for a culture-independent study of the identity of the transconjugants. The culture-dependent approach was also unsuccessful as the sorted transconjugants were unculturable under the given conditions (SWW, 30ºC). This is not surprising since the biggest fraction of wastewater and sludge bacterial community is unculturable (Irie et al., 2016; Muela et al., 2011), and many species co-depend on other bacteria for their survival (Bodor et al., 2020).

Unlike for pKJK5, the potential transfer of the narrow host range plasmid R64 towards the activated sludge community *in situ* could not be proved. The transferability of R64 plasmid towards activated sludge Enterobacteria *in vitro* (filter mating) has been previously stated (Li et al., 2018b), albeit results supporting those findings have not been published. The absence of quantifiable transconjugants might indicate a lack of transfer but can also be caused by quantification limits in the given setup. Enterobacteria in sewage occur in densities of up to 10^4 -10^6 CFUs mL^{-1} of influent (Novo and Manaia, 2010; Pallares-Vega et al., 2021) and 10^6 CFUs g TS^{-1} (roughly 10^3 CFUs mL^{-1}) in activated sludge (Pallares-Vega et al., 2021). The transfer rate of the R64 in solid agar and the liquid mating plasmid has been estimated in 10^{-3} transconjugants per recipient (Neil et al., 2020). This transfer rate combined with Enterobacteria concentration in the activated sludge might not have been sufficient to generate transconjugants. Alternatively, these transconjugants might have occurred below the limit of detection. Longer mating times than the one used in this study (24h) might provide room for new transfer events and clonal expansion of the transconjugants, overcoming the detection threshold. In this line, Mantilla-Calderon and Hong, (2017) observed transfer of narrow host range IncF plasmid towards other Enterobacteria (Shigella and Citrobacter genera) only after 72h of mating in an activated sludge

reactor. However, the tradeoff of using longer mating time is an increase in the inaccuracy for transfer rates assessment due to possible simultaneous clonal expansion of transconjugants.

5.4.2. The detection of transconjugants is hindered under psychrophilic temperatures and anaerobic conditions

Under aerobic conditions, fewer transconjugants were detected at 15°C than at 30°C, which can result from fewer transfer events and a reduced clonal expansion related to slower metabolic activity at psychrophilic temperatures. Under psychrophilic temperatures, the culture-independent assessment of plasmid transfer might also be hindered. A slower decay rate of donors at this temperature in comparison with 30°C might have hampered the actual quantification of transconjugant cells. From the total registered events (10^6), approximately 4-7 x10^5 corresponded to bacteria. From the latter, up to 50-92% and 3-6% accounted for donors at 15°C and 30°C, respectively (**Figure S5.2**). Faster decay of *E. coli* at higher temperature is in accordance with previous observations in water environments (Blaustein et al., 2013). The longer persistence of donors at low temperatures could be caused by a slower microbial metabolic activity leading to minor consumption of the resources and less competition with the indigenous microbiota. A decrease in protozoa predation at lower temperatures could be another reason for the more prolonged survival of donors. A bias in the sample processing might also have favoured the predominance of donor counts. Vigorous agitation and sonication in a water bath might have been insufficient to correctly disaggregate the indigenous cells attached to the sludge flocs, favouring the counts of planktonic cells (probably dominated by *E. coli* donors) instead.

The decay of donors was also lower in half of the anaerobic experiments at 30°C in comparison with the aerobic version of the same temperature. Shorter persistence of *E. coli* in aerobic conditions in comparison with anaerobic conditions has been previously observed in activated sludge microcosms (Jong et al., 2020; Mantilla-Calderon and Hong, 2017; Merlin et al., 2011), and could be due to a more intensive predatory pressure in aerobic environments (Jong et al., 2020). The presence of oxidative metabolic products or the preference of *E. coli* for oxygen-free environments has also been suggested as possible causes of this difference (Mantilla-Calderon and Hong, 2017).

Regarding the plasmid transfer, only two replicates at 30°C presented transconjugants above the limit of quantification by flow cytometry but were detected by plate counting in all the six replicates. This inconsistency can be due to the differences in detection limits between the two methods. Higher donor cell densities might have hampered the detection of

transconjugants by flow cytometry, as described for the temperature experiments. However, the discrepancy between methods might also result from a bias in the culture-based approach, as transconjugants could have originated during the culturing phase (Smit and Van Elsas, 1990). The background of indigenous resistant bacteria from activated sludge was 10-fold higher after anaerobic (ca. 10^3 CFUs mL^{-1}) than after aerobic incubation, maybe due to enrichment of certain taxa during the anaerobiosis process. A higher number of already resistant recipients might have facilitated the transfer within the culturing plate.

The literature on the influence of redox conditions on plasmid transfer is scarce, and results remain controversial. Merlin et al. (2011) have detected the transfer of another IncP-1 plasmid (pB10) towards the indigenous microbial community of digested sludge under anaerobic conditions. However, in a parallel experiment also in anaerobiosis but using activated sludge community instead, no transfer has been observed. Another recent study challenged sludge communities that were long term pre-conditioned to different redox regimes with an IncP-1 plasmid (RP4). Higher transfer rates during anaerobic conditions than in anoxic or aerobic conditions were found, presumably due to the more extended stability of the host under anaerobic conditions (Jong et al., 2020). Thus, a steady and mature anaerobic community might be more permissive towards the transfer of IncP-1 plasmids than the microbial community of activated sludge under transitory anaerobiosis. Whether narrow host range plasmids would follow a similar dynamic is still unknown. Only one study based on IncF plasmids has approached this question, finding no transconjugants in anaerobic sludge microcosms (Mantilla-Calderon and Hong, 2017). Because of time constraints, the transfer of IncI R64 plasmid under anaerobic conditions could not be assessed in this study. Conjugal transfer of IncI plasmids under anoxic conditions has been reported in an *in vitro* model based on *E. coli* as both donor and recipient (Anjum et al., 2018), albeit at low transfer rates (T/D 10^{-4}). Recently, Neil and collaborators have also proved *in situ* transfer from narrow host range plasmids (IncF and IncI) towards gut community under hypoxic conditions in mice. Their experiments have proven only the transfer of some specific plasmids (including R64) and have shown that the transfer rates are low (10^{-5} – 10^{-3}) (Neil et al., 2020). Thus, the transfer of narrow host range plasmids towards single species and mixed communities under anoxic conditions is possible. Yet, potential transfer towards complex communities in which putative Enterobacteriaceae receptors are not predominant (i.e. activated sludge holds 10^5 fold fewer *E. coli* than in gut microbiota) (García-Aljaro et al., 2019) might fall below the limit of quantification of culture-dependent and independent methods.

5.5. Conclusions

In the present study, we evaluated the potential transfer of a broad and a narrow host range plasmid in activated microcosm mimicking common conditions within the biological treatment, such as different oxygen regimes and psychrophilic temperatures. The main conclusions from this work are:

- Transfer of the broad host range plasmid pKJK5 from *E. coli* towards bacterial populations of the activated sludge community was detected, unlike for the narrow host range plasmid R64.
- Culture-independent measurement of transconjugants from microcosm settings by flow cytometry is possible. However, low transconjugants numbers might prevent the use of cell sorting for subsequent, direct identification of transconjugants by 16S rRNA gene amplicon sequencing.
- The persistence of donor cells at 15°C and under anaerobic conditions hindered detecting and quantifying transconjugants by flow cytometry.
- Further evaluation of the transfer dynamics of alternative IncI plasmids and other relevant narrow host range plasmids (IncF, IncA/O) would be fundamental to unravel the role of these vectors in disseminating antibiotic resistance determinants in activated sludge.

5.6. Acknowledgements

This work was performed in the cooperation framework of Wetsus, European Centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces. This work has also received funding from STOWA and from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie [grant agreement No. 665874]. The authors like to thank the participants of the Wetsus research theme Source Separated Sanitation for the fruitful discussions and their financial support.

The authors gratefully acknowledge the operators at the WWTP Lyngby-Taarbæk for their help in the sample collection and to Gonçalo Macedo for his assistance during the experimental work.

5.7. Supplementary information

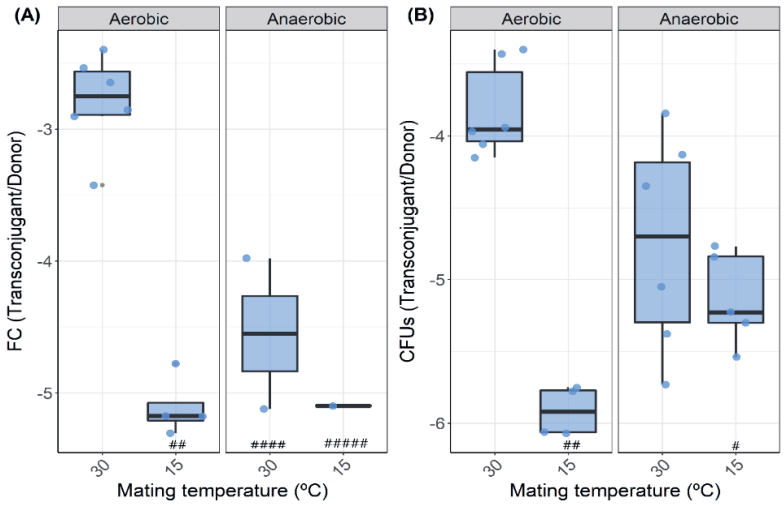
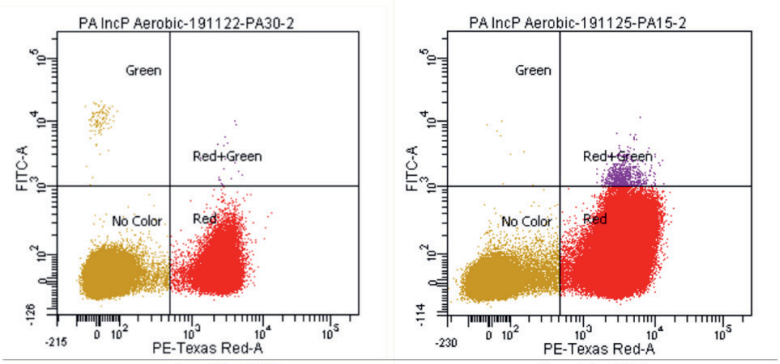


Figure S5.1. Transfer rates of pKJK5 under aerobic and anaerobic conditions measured by flow cytometry or plate counting in selective media. "#" stands for each replicate without detectable transconjugants or transconjugants below the quantification limit.



	Aerobic 30°C			Aerobic 15°C		
	Events	**%**	**% Bacteria**	**Events**	**%**	**% Bacteria**
All events	1000000	-	-	1000000	-	-
Bacteria	469362	46.9	-	686129	68.6	-
No color (AS com)	442347	-	94.2	204249	-	29.8
Red (Donor)	26888	-	5.7	481132	-	70.1
Green (Tc)	108	-	0.0	8	-	0.0
Red + Green (*)	19	-	0.0	740	-	0.1

Figure S5.2. Flow cytometry scatter plots and events summary for two single replicates of pKJK5 transfer towards activated sludge community under aerobic conditions and optimal temperature (30°C) and environmental temperature (15°C). Cells expressing Red + Green (*): Repression of GFP is not achieved in some donors, and thus, they express both mCherry and GFP fluorescence simultaneously.

5.8. References

Anjum, M., Madsen, J.S., Espinosa-Gongora, C., Jana, B., Wiese, M., Nielsen, D.S., Sørensen, S.J., Moodley, A., Bortolaia, V., Guardabassi, L., 2018. A culture-independent method for studying transfer of IncI1 plasmids from wild-type Escherichia coli in complex microbial communities. J. Microbiol. Methods 152, 18–26. https://doi.org/10.1016/j.mimet.2018.07.009

Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J. V, 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 14, 176–82. https://doi.org/10.1016/j.tim.2006.02.006

Bellanger, X., Guilloteau, H., Bonot, S., Merlin, C., 2014a. Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review. Sci. Total Environ. 493, 872–882. https://doi.org/10.1016/j.scitotenv.2014.06.070

Bellanger, X., Guilloteau, H., Breuil, B., Merlin, C., 2014b. Natural microbial communities supporting the transfer of the IncP-1β plasmid pB10 exhibit a higher initial content of plasmids from the same incompatibility group. Front. Microbiol. 5, 1–8. https://doi.org/10.3389/fmicb.2014.00637

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439

Blaustein, R.A., Pachepsky, Y., Hill, R.L., Shelton, D.R., Whelan, G., 2013. Escherichia coli survival in waters: Temperature dependence. Water Res. 47, 569–578. https://doi.org/10.1016/j.watres.2012.10.027

Bodor, A., Bounedjoum, N., Vincze, G.E., Erdeiné Kis, Á., Laczi, K., Bende, G., Szilágyi, Á., Kovács, T., Perei, K., Rákhely, G., 2020. Challenges of unculturable bacteria: environmental perspectives. Rev. Environ. Sci. Biotechnol. 19, 1–22. https://doi.org/10.1007/s11157-020-09522-4

Bonot, S., Merlin, C., 2010. Monitoring the dissemination of the broad-host-range plasmid pB10 in sediment microcosms by quantitative PCR. Appl. Environ. Microbiol. 76, 378–382. https://doi.org/10.1128/AEM.01125-09

Cai, L., Ju, F., Zhang, T., 2014. Tracking human sewage microbiome in a municipal wastewater treatment plant. Appl. Microbiol. Biotechnol. 98, 3317–3326. https://doi.org/10.1007/s00253-013-5402-z

Carattoli, A., 2013. Plasmids and the spread of resistance. Int. J. Med. Microbiol. 303, 298–304. https://doi.org/10.1016/j.ijmm.2013.02.001

Clesceri, L.S., Rica, E.W., Baird, R.B., Eaton, A.D., 1998. No Title, in: Standard Methods for the Examination of Water and Wastewater. American Public Health Association, American Water Works Association and Water Environment Federation, Washington D.C.

Francino, M.P., 2016. Antibiotics and the human gut microbiome: Dysbioses and accumulation of resistances. Front. Microbiol. 6, 1–11. https://doi.org/10.3389/fmicb.2015.01543

García-Aljaro, C., Blanch, A.R., Campos, C., Jofre, J., Lucena, F., 2019. Pathogens, faecal indicators and human-specific microbial source-tracking markers in sewage. J. Appl. Microbiol. 126, 701–717. https://doi.org/10.1111/jam.14112

Geisenberger, O., Ammendola, A., Christensen, B.B., Molin, S., Schleifer, K.H., Eberl, L., 1999. Monitoring the conjugal transfer of plasmid RP4 in activated sludge and in situ identification of the transconjugants. FEMS Microbiol. Lett. 174, 9–17. https://doi.org/10.1016/S0378-1097(99)00114-7

Irie, K., Fujitani, H., Tsuneda, S., 2016. Physical enrichment of uncultured Accumulibacter and Nitrospira from activated sludge by unlabeled cell sorting technique. J. Biosci. Bioeng. 122, 475–481. https://doi.org/10.1016/j.jbiosc.2016.03.020

Jacquiod, S., Brejnrod, A., Morberg, S.M., Abu Al-Soud, W., Sørensen, S.J., Riber, L., 2017. Deciphering conjugative plasmid permissiveness in wastewater microbiomes. Mol. Ecol. 26, 3556–3571. https://doi.org/10.1111/mec.14138

Jensen, K.F., 1993. The Escherichia coli K-12 "wild types" W3110 and MG1655 have an rph frameshift mutation that leads to pyrimidine starvation due to low pyrE expression levels. J. Bacteriol. 175, 3401–7.

Jong, M.C., Harwood, C.R., Blackburn, A., Snape, J.R., Graham, D.W., 2020. Impact of Redox Conditions on Antibiotic Resistance Conjugative Gene Transfer Frequency and Plasmid Fate in Wastewater Ecosystems. Environ. Sci. Technol. 54, 14984–14993. https://doi.org/10.1021/acs.est.0c03714

Kiragosyan, K., Picard, M., Sorokin, D.Y., Dijkstra, J., Klok, J.B.M., Roman, P., Janssen, A.J.H., 2020. Effect of dimethyl disulfide on the sulfur formation and microbial community composition during the biological H2S removal from sour gas streams. J. Hazard. Mater. 386, 121916. https://doi.org/10.1016/j.jhazmat.2019.121916

Klümper, U., Droumpali, A., Dechesne, A., Smets, B.F., 2014. Novel assay to measure the plasmid mobilizing potential of mixed microbial communities. Front. Microbiol. 5, 1–9. https://doi.org/10.3389/fmicb.2014.00730

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945. https://doi.org/10.1038/ismej.2014.191

Li, L., Dechesne, A., He, Z., Madsen, J.S., Nesme, J., Sørensen, S.J., Smets, B.F., 2018a. Estimating the Transfer Range of Plasmids Encoding Antimicrobial Resistance in a Wastewater Treatment Plant Microbial Community. Environ. Sci. Technol. Lett. 5, 260–265. https://doi.org/10.1021/acs.estlett.8b00105

Li, L., Dechesne, A., Madsen, J.S., Nesme, J., Olesen, A.K., Sorensen, S., Smets, B.F., 2018b. Transfer and long-term persistence of plasmids encoding antimicrobial resistance in wastewater treatment plant microbial communities, in: 17th International Symposium on Microbial Ecology, Leipzing, Germany.

Li, L., Dechesne, A., Madsen, J.S., Nesme, J., Sørensen, S.J., Smets, B.F., 2020. Plasmids persist in a microbial community by providing fitness benefit to multiple phylotypes. ISME J. 14, 1170–1181. https://doi.org/10.1038/s41396-020-0596-4

Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I.C., Zammit, I., Kampouris, I., Vaz-Moreira, I., Nunes, O.C., 2018. Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environ. Int. 115, 312–324. https://doi.org/10.1016/j.envint.2018.03.044

Mantilla-Calderon, D., Hong, P.-Y., 2017. Fate and Persistence of a Pathogenic NDM-1-Positive Escherichia coli Strain in Anaerobic and Aerobic Sludge Microcosms. Appl. Environ. Microbiol. 83, 1–13. https://doi.org/10.1128/AEM.00640-17

Merlin, C., Bonot, S., Courtois, S., Block, J.-C., 2011. Persistence and dissemination of the multiple-antibiotic-resistance plasmid pB10 in the microbial communities of wastewater sludge microcosms. Water Res. 45, 2897–905. https://doi.org/10.1016/j.watres.2011.03.002

Muela, A., Orruño, M., Alonso, M.L., Pazos, M., Arana, I., Alonso, R.M., Jiménez, R.M., Garaizabal, I., Maguregui, M.I., Barcina, I., 2011. Microbiological parameters as an additional tool to improve wastewater treatment plant monitoring. Ecol. Indic. 11, 431–437. https://doi.org/10.1016/j.ecolind.2010.06.014

Neil, K., Allard, N., Grenier, F., Burrus, V., Rodrigue, S., 2020. Highly efficient gene transfer in the mouse gut microbiota is enabled by the IncI2 conjugative plasmid TP114. Commun. Biol. 3. https://doi.org/10.1038/s42003-020-01253-0

Novo, A., Manaia, C.M., 2010. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. Appl. Microbiol. Biotechnol. 87, 1157–1166. https://doi.org/10.1007/s00253-010-2583-6

Pallares-Vega, R., Hernandez Leal, L., Fletcher, B.N., Vias-Torres, E., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2021a. Annual dynamics of antimicrobials and resistance determinants in flocculent and aerobic granular sludge treatment systems. Water Res. 190, 116752. https://doi.org/10.1016/j.watres.2020.116752

Pallares-Vega, R., Macedo, G., Brouwer, M.S.M., Hernandez Leal, L., van der Maas, P., van Loosdrecht, M.C.M., Weissbrodt, D.G., Heederik, D., Mevius, D., Schmitt, H., 2021b. Temperature and Nutrient Limitations Decrease Transfer of Conjugative IncP-1 Plasmid pKJK5 to Wild Escherichia coli Strains. Front. Microbiol. 12. https://doi.org/10.3389/fmicb.2021.656250

Perry, J.A., Wright, G.D., 2013. The antibiotic resistance "mobilome": Searching for the link between environment and clinic. Front. Microbiol. 4, 1–7. https://doi.org/10.3389/fmicb.2013.00138

Pinilla-Redondo, R., Cyriaque, V., Jacquiod, S., Sørensen, S.J., Riber, L., 2018. Monitoring plasmid-mediated horizontal gene transfer in microbiomes: recent advances and future perspectives. Plasmid 99, 56–67. https://doi.org/10.1016/j.plasmid.2018.08.002

Pinilla-Redondo, R., Olesen, A.K., Russel, J., de Vries, L.E., Christensen, L.D., Musovic, S., Nesme, J., Sørensen, S.J., 2020. Conjugative dissemination of plasmids in rapid sand filters: a trojan horse strategy to enhance pesticide degradation in groundwater treatment. https://doi.org/10.1101/2020.03.06.980565

Quintela-Baluja, M., Abouelnaga, M., Romalde, J., Su, J.Q., Yu, Y., Gomez-Lopez, M., Smets, B., Zhu, Y.G., Graham, D.W., 2019. Spatial ecology of a wastewater network defines the antibiotic resistance genes in downstream receiving waters. Water Res. 162, 347–357. https://doi.org/10.1016/j.watres.2019.06.075

R Core Team, 2018. R: A language and environment for statistical computing. R Found. Stat. Comput. Vienna.

Rankin, D.J., Rocha, E.P.C., Brown, S.P., 2011. What traits are carried on mobile genetic elements, and why? Heredity (Edinb). 106, 1–10. https://doi.org/10.1038/hdy.2010.24

Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Guerra, B., Mevius, D.J., Hordijk, J., 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J. Antimicrob. Chemother. 73, 1121–1137. https://doi.org/10.1093/jac/dkx488

Sampei, G. ichi, Furuya, N., Tachibana, K., Saitou, Y., Suzuki, T., Mizobuchi, K., Komano, T., 2010. Complete genome sequence of the incompatibility group I1 plasmid R64. Plasmid 64, 92–103. https://doi.org/10.1016/j.plasmid.2010.05.005

Seoane, J., Yankelevich, T., Dechesne, A., Merkey, B., Sternberg, C., Smets, B.F., 2011. An individual-based approach to explain plasmid invasion in bacterial populations. FEMS Microbiol. Ecol. 75, 17–27. https://doi.org/10.1111/j.1574-6941.2010.00994.x

Smit, E., Van Elsas, J.D., 1990. Determination of Plasmid Transfer Frequency in Soil: Consequences of Bacterial Mating on Selective Agar Media. Curr. Microbiol. 21, 151–157.

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., 2005. Studying plasmid horizontal transfer in situ: a critical review. Nat. Rev. Microbiol. 3, 700–710. https://doi.org/10.1038/nrmicro1232

Yang, D., Wang, J., Qiu, Z., Jin, M., Shen, Z., Chen, Z., Wang, X., Zhang, B., Li, J.-W., 2013. Horizontal transfer of antibiotic resistance genes in a membrane bioreactor. J. Biotechnol. 167, 441–7. https://doi.org/10.1016/j.jbiotec.2013.08.004

CHAPTER 6

General discussion and Outlook

6.1. Foreword.

This thesis presents a comprehensive evaluation of the dynamics of antibiotic and antibiotic resistance determinants in wastewater and wastewater treatment systems. Throughout **Chapters 2** and **3**, we provided quantitative data on the occurrence, removal of antibiotics and resistance determinants. In **Chapters 2** and **3**, we also investigated the possible factors affecting their occurrence and removal and quantified the impact of the relevant factors. In addition, in **Chapters 4** and **5** we assessed and quantified horizontal exchange rates of mobile genetic elements associated with resistance determinants in relevant conditions for wastewater systems. Ultimately, this work provides insights into antimicrobial resistance in Dutch sewage and sewage treatment. The data presented here can be used to develop mitigation strategies and policies to tackle the antibiotic resistance challenge at both WWTP and environmental levels.

A discussion of the results addressing each of the research questions and objectives proposed for this thesis is given hereafter. The main findings obtained throughout the thesis are also placed in the context of the current literature, and future perspectives and research questions for further investigations are provided.

6.2. Antibiotics and antibiotic resistance in sewage in the country with the lowest antibiotic consumption in the EU.

For the last 10 years, The Netherlands has been the country with the lowest antibiotic consumption in the European Union (10 Defined Daily Dose (DDD) per 1000 inhabitants) for both community and hospital ranks (ESAC-Net, 2018). The Netherlands is also among the European countries with the lowest prevalence of resistance among clinical isolates (ECDC, 2018). Despite such close monitoring of antibiotics and antibiotic resistance in the clinical context, there was limited knowledge of the presence of these agents in the Dutch environment in general and sewage system in particular.

In **Chapter 3,** up to 10 (azithromycin, clarithromycin, ofloxacin, sulfamethoxazole, sulphapyridine, trimethoprim, doxycycline, tetracycline and clindamycin) of the 24 tested antibiotics compounds were frequently detected in the Duch influent, in concentrations ranging from 10ng to 1 µg per L. When compared to Spain, a country with a high antibiotic consumption within the European Union (ESAC-Net, 2018), similar concentrations were reported (**Table 6.1**). This might seem surprising, as the consumption of antibiotics in Spain (ca. 26 DDD) is more

than double of the Dutch consumption (ca. 10 DDD). However, the large difference is mainly caused by the greater use of penicillins in Spain (14 vs 3 DDD). The latter is not reflected in the sewer because penicillins are unstable in aqueous systems and, consequently, rarely detected in water (van Krimpen et al., 1987). The predominant antibiotics in the influent of both countries were the fluoroquinolones ciprofloxacin and ofloxacin, accounting for only 11-12% of the consumption in both countries (ESAC-Net, 2018). Higher use of tetracyclines (doxycycline and tetracycline) in The Netherlands or cephalosporins (i.e., cefotaxime) in Spain were reflected in frequency of detection of such compounds in the influent.

Table 6.1. Antibiotic residues detected in the influent of WWTPs in The Netherlands (NL) compared to Spain (ES). [1]: Chapter 3; [2]:(Gracia-Lor et al., 2012); [3] (Rodriguez-Mozaz et al., 2015). Antibiotic abbreviation AZI: Azithromycin; CLAR: Clarithromycin; CIP: Ciprofloxacin; OFX: Ofloxacin; SMO: Sulfamethoxazole; TRIM: Trimethoprim; DOX: Doxycycline; TET: Tetracycline; CM: Clindamycin; CFT: Cefotaxime. (*): CFT Results of NL based on only 2 positive samples out of 36.

		Compound (µg L^{-1})										Ref.
		AZI	CLAR	CIP	OFX	SMO	SUPY	TRIM	DOX	TET	CM	CFT	
NL	Min	<LOD	<LOD	0,22	<LOD	<LOD	0,05	<LOD	0,24	<LOD	<LOD	<LOQ	[1]
	Median	0,39	0,10	0,84	0,10	0,36	0,42	0,20	0,47	0,29	0,02	0,20*	
	Max	0,96	0,28	1,54	1,40	0,98	0,87	0,32	0,92	0,83	0,03	0,27*	
ES	Min	na	0,13	1,21	0,29	<LOQ	na	0,06	na	na	na	na	[2]
	Median	na	0,23	2,45	0,76	0,28	na	0,10	na	na	na	na	
	Max	na	0,62	3,85	0,96	0,54	na	0,16	na	na	na	na	
ES	Min	0,19	0,46	0,64	0,58	0,33	<LOD	0,09	<LOD	<LOD	na	0,25	[3]
	Max	0,46	0,55	1,31	1,56	0,41	0,05	0,18	<LOD	<LOD	na	0,36	

Thus, consumption patterns of antibiotics were reflected in sewage (presence/absence) except for the penicillins group, in accordance with their physicochemical behaviour. Moreover, similar antibiotic residues were present in Dutch and Spanish wastewater despite their different European rank classification for antibiotic consumption (ESAC-Net, 208).

For the assessment of antibiotic resistance-related genes in sewage (**Chapters 2** and **3**), we targeted 6 ARGs (*ermB, sul1, sul2, tetM, qnrS,* and bla_{CTX-M}) as a representative selection of resistance to commonly used antibiotics (Berendonk et al., 2015) and 2 MGEs (*intI1* and *korB* –for IncP-1 plasmids). ARGs and MGEs occurred within different concentrations in influent, ranging from 10^4 to 10^7 gene copies per mL of sewage, in the following order of prevalence (*ermB*/*sul1*/*intI1* > *sul2* > *tetM*/*korB* > *qnrS* > bla_{CTX-M}). Results were consistent among the cross-sectional study in over 60 WWTPs (**Chapter 2**) and the long-term study in 3 WWTPs (**Chapter**

3). In **Chapter 2** we provided a comparative overview with other international studies in **Table S3**. A subtract from this table is now displayed as **Table 6.2**. The concentrations observed in the Netherlands were often equivalent to those described for Tunisia or Switzerland. Yet, similarities varied per gene (i.e., concentrations of *ermB* more comparable than *qnrS*), perhaps conditioned by distinct antibiotic consumption patterns across countries. Recently Pärnänen et al. (2019) reported a gradient in the cumulative relative abundance of ARGs following the European antibiotic consumption pattern across six European countries. However, this statement should be analyzed carefully. Overall, Portuguese, Spanish and Cypriot samples (high antibiotic consumption) had a higher relative abundance of resistant genes than German, Finish or Norwegian influent (low antibiotic consumption), but mainly driven by the high occurrence found in the Portuguese sewage. Moreover, some ARGs families (i.e., tetracyclines, aminoglycosides) were more abundant in countries with low antibiotic consumption, in line with their higher use. In contrast, Hendriksen et al. (2019) found that consumption data explained only a minor part of the differences in ARGs variation across the sewer in a metagenome based global monitoring of the sewage resistome. Instead, the authors report that the socio-economic and healthcare differences were the main predictors for ARGs variations across countries.

Table 6.2. ARGs concentrations in influent samples from different countries. Based on table S1.3. Standard deviation is provided for the studies with more than 1 WWTP. Countries abbreviation: NL: Netherlands, TN: Tunisia, IT: Italy; PL: Poland; ES: Spain; CH: Spain.

Reference	Location	Nb WWTPs	Sample type	Average absolute log_{10} gene copies mL^{-1}								
				16S	*ermB*	*sul 1*	*sul 2*	*tetW*	*tetM*	*qnrS*	*bla* CTX-M	*intI1*
Chapter II	NL	62	I	8,52	6,48	6,62	5,71		5,66	5,44	4,40	6,53
			SD	*0,40*	*0,33*	*0,29*	*0,24*		*0,29*	*0,42*	*0,28*	*0,33*
Rafraf et al., 2016	TN	5	I	8,53	6,27	6,84				4,12	4,21	6,52
			SD	*0,16*	*0,71*	*0,42*				*0,75*	*1,20*	*0,39*
Di Cesare et al., 2016	IT	3	I		6,13		5,54			6,26		5,72
			SD		*0,39*		*0,16*			*0,34*		*0,14*
Makowska et al., 2016	PL	1	I	8,66		4,88	4,41		3,00			4,36
Wen et al., 2016	CN	4	I	8,46		5,70	6,99	6,69			3,40	6,60
			SD	*0,14*		*0,20*	*0,21*	*0,26*			*0,55*	*0,13*
Rodriguez-Mozaz et al., 2015	ES	1	I	7,48	6,00	5,70		6,00		5,00		
Czekalski et al., 2014	CH	1	I	8,34		7,00	6,29	5,27	6,15			

A reduction of antibiotic consumption is recommended to prevent the emergence of multi-resistant isolates and new resistant variants (WHO, 2017). Yet, complying with this maxima does not guarantee a lower prevalence of ARGs in the patient's microbiota (Merlin, 2020), and may explain the lack of more significant differences in the sewage. Another possible cause for such "homogenization" is the sewage system itself. The change from gut anaerobic environments towards microaerophilic or aerobic in sewage (along with other conditions) is known to shape the microbial communities selecting those microorganisms better adapted to such environments (Quintela-Baluja et al., 2019). Indeed, recently, a remarkable decline in ESBL genes (higher than during wastewater treatment) has been observed during sewage conveyance (Li et al., 2021). Whether different sewer designs and distances impacting the transit time until the WWTP would significantly affect the degree of decline and change remains unknown and is worth exploring.

6.3. Discharge of antibiotics and resistance determinants from wastewater treatments.

6.3.1. Dissemination through effluent discharges.

In addition to surveillance on the incoming sewage, it is fundamental to determine the concentrations of both antibiotic and antibiotic resistant determinants after the wastewater treatment to calculate their emission levels towards the environment.

In **Chapter 2** we observed that most of the antibiotics found in influent were still detected in the effluent (except for ofloxacin and doxycycline). Generally, the compounds were found at a lower concentration than in the influent (ca. 20-400 ng L^{-1}), indicating a partial reduction by the water treatment (10-100%). Fluoroquinolones and macrolides were the most prevalent compounds, and the registered concentrations were comparable to that obtained for the Spanish studies (except for ofloxacin), as described in **Table 6.3**. In contrast, a recent multinational surveillance study across Europe pointed to a higher antibiotic presence in effluents in countries with high antibiotic consumption (Portugal, Spain or Ireland) in comparison to countries with low antibiotic consumption (Germany, Norway, Finland) (Rodriguez-Mozaz et al., 2020). However, these conclusions were based on the use of cumulative concentrations, which is deceiving. Moreover, the data presented in the same study showed that patterns differed per antibiotic. For instance, while ciprofloxacin was 2 to 10-fold higher in countries with high antibiotic consumption, the cephalosporin cefalexin concentration was 4 to 10-fold higher in Finish effluents than in those of Portugal, Spain or Cyprus. A similar comparison holds for

other antibiotics on the list.

Hence, despite a partial reduction by the wastewater treatment, some antibiotics are still discharged towards the environment with comparable concentration ranges across EU countries with different antibiotic consumption patterns.

For particular antibiotics (i.e. ciprofloxacin and azithromycin), such concentrations often exceed the PNEC-MIC levels (Chapter 3, Carvalho and Santos, 2016; Rodriguez-Mozaz et al., 2020) and might act as selectors in the receiving waters. The associated risk (adverse effect on target organisms, ie., selection for resistant strains) of such discharges would be ultimately dependent on the dilution factor of the receiving waterbody. In the Netherlands, the predicted median dilution factor is estimated to be 18, which is rather low (for context, Spain: 26, Norway: 2453.29) (Keller et al., 2014). A preliminary risk assessment (**Table 6.3.**) based on the Risk Quotients (RQs) was calculated from the median concentrations obtained in Chapter 3, the dilution factor and the equations proposed by Rodriguez-Mozaz et al. (2020) and the European Community guidelines (EC TGD, 2003) **Eq.(6.1)** and **Eq. (6.2).**

Eq. (6.1): $PEC = (MC)/(DF)$ **Eq. (6.2):** $RQ = (PEC)/(PNEC\text{- }MIC)$

Where PEC is the Predicted environmental concentration, MC is Measured concentration (based on average values in Chapter 3), and DF is the dilution factor for The Netherlands (18) based on (Keller et al., 2014). Finally, PNEC-MIC values from (Bengtsson-Palme and Larsson, 2016) are used to calculate the Risk Quotidien (RQ).

Table 6. 3. Antibiotic residues detected in the effluent of WWTPs The Netherlands (NL) and compared to Spain (ES). [1]: Chapter 3; [2]:(Gracia-Lor et al., 2012); [3] (Rodriguez-Mozaz et al., 2015). Underlined and in italic concentrations above the PNEC-MIC for each compound. In bold and italic RQ >1 representing a high risk. Antibiotic abbreviation AZI: Azithromycin; CLAR: Clarithromycin; CIP: Ciprofloxacin; OFX: Ofloxacin; SMO: Sulfamethoxazole; TRIM: Trimethoprim; DOX: Doxycycline; TET: Tetracycline; CM: Clindamycin; CFT: Cefotaxime.

		Compound (µg L^{-1})											Ref
		AZI	**CLAR**	**CIP**	**OFX**	**SMO**	**SUPY**	**TRIM**	**DOX**	**TETR**	**CM**	**CFT**	
	PNEC-MIC	**0.25**	**0.25**	**0.06**	**0.50**	**16.00**	**na**	**0.50**	**2.00**	**1.00**	**1.00**	**0.13**	
NL	Min	0.07	0.01	*0.06*	na	0.07	0.03	0.02	<LOQ	0.06	0.01	<LOQ	[1]
	Median	*0.26*	0.05	*0.11*	na	0.14	0.15	0.10	<LOQ	0.08	0.03	<LOQ	
	Max	*0.79*	0.19	*0.29*	na	0.33	0.28	0.14	<LOQ	0.12	0.01	0.02	
	RQ	0.06	0,01	***1.83***	na	<0.01	na	0.01	na	<0.01	<0.01	0.01	
ES	Min	na	0.01	*0.52*	0.09	<LOQ	na	0.06	na	na	0.01	na	[2]
	Median	na	0.02	*0.70*	0.13	0.10	na	0.09	na	na	0.02	na	
	Max	na	0.06	*1.08*	0.15	0.12	na	0.10	na	na	0.02	na	
ES	Min	<LOQ	0.09	*0.11*	0.08	0.06	<LOQ	0.01	<LOQ	<LOQ	<LOQ	*0.21*	[3]
	Max	0.22	0.12	*0.18*	*1.17*	0.08	0.05	0.13	<LOQ	<LOQ	<LOQ	*0.23*	

While the dilution effect reduced the risk for azithromycin (only sporadically high concentrated discharges might represent moderate risk: 0.1 < RQ <1), the current discharge levels of ciprofloxacin encompass a constant high risk (RQ> 1) in the Netherlands. This risk, of course, refers to single compound effects and does not account for other putative synergistic effects caused by the co-occurrence of several antibiotics (as is the case in natural ecosystems), for which the PNEC-MIC might be lower.

Regarding the gene determinants, all the tested genes were also found in the effluent, except for *bla*$_{CTX-M}$ undetected in up to 10-40% of the samples. Results were once more consistent among the cross-sectional and the long-term study, as observed for the influent. In the effluent, the average absolute concentration of ARGs (62 WWTPs) range from the minimal 9.1 x 10^1 (*bla*$_{CTX-M}$) and maximal of 7 x 10^4 (*sul1*) and for the MGEs ca. 5 x10^4 with the following order of prevalence (intI1/*sull1/korB* > *sul2* > *tetM*/*qnrS* > *bla*$_{CTX-M}$). For the ARGs, these absolute concentrations are comparable to those reported in Italian and Spanish studies (Di Cesare et al., 2016; Rodriguez-Mozaz et al., 2015), and 1 to 3 logs lower than those described for Tunisian WWTPs (Rafraf et al., 2016).

These concentrations indicate that Dutch WWTPs discharge from 10^4 to 10^6 gene copies of each ARG per L to the receiving surface waters, comprising ~10^{11} to 10^{13} gene copies discharged per day per WWTP (based on a middle-sized WWTP treating 50.000 m3 day). The impact of such emissions on the surface waters is again case-dependent, conditional on the dilution factor and the receiving waterbody's basal resistome (LaPara et al., 2011; LaPara et al., 2015). In the Netherlands, a significant increase in the ARGs concentration downstream the WWTP in comparison to upstream has been reported (Sabri et al., 2018). Similar observations have been made for antibiotic-resistant bacteria (Verburg et al., 2019), albeit the persistence of these agents along the water pathway is still under study (van Heijnsbergen et al., in preparation).

The associated risks (in this case, to human health) are challenging to estimate. The risk will depend on the inactivation throughout the water pathway (which might vary per catchment (Lee et al., 2021)), the input from other sources (Blaak et al., 2015), and the frequency of contact and volumes of water ingestion (Leonard et al., 2015).

6.3.2. Sludge as a sink for some antibiotic residues and ARGs.

Activated sludge, formed by microbial aggregates, constitutes the core of the biological treatment, but it is also a by-product of wastewater treatment. Activated sludge acted as a sink

for several antibiotics (i.e., macrolides and tetracyclines) and for disinfectants (Benzalkonium chloride 12 and 14). These compounds were found even more frequently after anaerobic digestion, in ranges of 0.5-4.5 mg kg TS^{-1} for the antibiotics and 2-12 mg kg TS^{-1} for the disinfectants. Activated sludge (both flocculent and granular) also accumulated ARGs, in general following the concentration rank observed in influent. Only *ermB* presented lower relative abundance in granular sludge than in flocculent sludge. Similar observations have been reported recently (Sabri et al., 2018), perhaps related to the limited integration of bacteria carrying *ermB* (i.e. *Lactobacillales*) in the granules' microbial community (Ali et al., 2019).

Some genes were highly reduced by the digestion process (*qnrS*, bla_{CTXM}), while others increased their relative abundance (*ermB* and especially *tetM*), which seems in line with observations in bench-scale reactors (Ma et al., 2011). Shifts in the microbial community during anaerobic digestion are most likely causing these dynamics. However, the accumulation of tetracyclines within the sludge might also select tetracycline resistant bacteria and promote horizontal gene transfer of plasmid-borne *tet* genes. Enhancement of conjugal transfer of plasmids *in vitro* or selection of the resistant transconjugants by sub-inhibitory concentrations of tetracycline (10 ug L^{-1}) have been observed (Jutkina et al., 2016). Sub-inhibitory concentrations of tetracyclines and other antibiotics (i.e. macrolides, lincosamides, and streptogramins) have also been reported to promote the conjugal transfer of the *tetM*-associated transposon Tn916 (Scornec et al., 2017). Since tetracyclines (tetracycline but also doxycycline) are strongly sorbed to the sludge fraction, concentrations above selective levels are more likely to occur in the sludge than in the effluent. Albeit, the bioavailability of these compounds within the sludge will also be determining their selective effects. Accordingly, further research addressing both the bioavailability of tetracyclines in the sludge as well as their potential effect on conjugation in sludge systems is needed. However, previous efforts in soil have emphasised the challenges of detecting such an effect (Schmitt et al., 2006)

6.4. Removal of resistance determinants in wastewater systems

6.4.1. Wastewater treatment systems are the solution, not the problem:

Wastewater treatment systems have been long regarded as hotspots for disseminating and increasing antibiotic resistance due to their unique characteristics: incoming antibiotic resistance gene carrying fecal bacteria, sub-inhibitory (but likely selective) concentrations of antibiotics (among other selectors), and high cell densities ideal for genetic exchange (Manaia et al., 2018). Moreover, conventional WWTPs are designed to remove organics and nutrients

rather than bacteria (and their ARGs) (Henze et al., 2005). However, in **Chapters 2** and **3** of this thesis, we showed a significant reduction of the absolute concentration of all the genes, including 6 ARGs and 2 MGEs by Dutch WWTPs. The average removals for the ARGs as stated in **Chapter 2** based on >60 WWTPs ranged from 1.72 logs (*sul1*) up to 2.58 logs (*ermB*) which correspond to a decrease of 98% to 99.7% of the tested ARGs. These findings are in agreement with (Yang et al., 2014), who reported removal of 99,8% revealed by a metagenomic approach which studied the dynamics of up to 271 ARG subtypes.

Beyond the removal of the absolute ARGs abundance, it is often discussed whether WWTPs are responsible for selecting resistant bacteria. This claim is sustained on the increase of relative abundance (related to the whole community) of resistant bacteria or ARGs after treatment. Such an effect has been observed for single ARGs in previous works (Rafraf et al., 2016; Rodriguez-Mozaz et al., 2015). From the results of both **Chapter 2** and **Chapter 3**, we can conclude that Dutch WWTPs did not increase the relative abundance of the tested genes. For most WWTPs, either no significant changes (for *sul1* or *sul2*) or even a significant decrease (for *ermB, tetM,* $bla_{CTX\text{-}M}$) was observed. Comparable trends were also registered for the cumulative relative abundance of most of the ARGs classes in other European countries (Pärnänen et al., 2019). Consequently, we can conclude that although conventional WWTPs are not designed to remove bacteria or ARGs, if well managed, they substantially reduce their absolute concentration and do not increase their relative abundance.

6.4.2. ARGs removal is achieved in the biological treatment:

The mechanisms for the removal of ARGs throughout the treatment are difficult to decipher. In **Chapters 2** and **3** we studied possible plant designs or operational conditions but no specific configuration comprised a significant improvement. In **Chapter 3** we evaluated the role of different stages and concluded that no significant removal of ARGs was observed for the primary clarification (**Figure 6.1**). These results are in line with the lack of significant differences in removing ARGs among WWTPs with or without primary treatment observed in **Chapter 2**. Thus, discarding the contribution of the primary clarifiers, we can conclude that the removal of ARGs was achieved throughout the biological treatment and secondary clarification. Even short contact time (short HRT) with the activated sludge, such as in the A stage of AB system (and its subsequently clarification step), already led to a significant (although moderate) reduction of ARGs (**Figure 6.1**). Longer HRT within the biological basin (B stage, CAS) seem to be necessary to achieve higher percentages of reduction (**Chapter 3**). Yet, across many plants, no consistent

effect of a longer HRT was observed to improve the elimination of ARGs (**Chapter 2**) significantly. We hypothesize that the optimal HRT is likely specific for each WWTP.

The exact pathways for ARG reduction within the biological treatment are still unclear. Adhesion to the activated sludge (that will settle in the secondary clarifier), out-competition by native microbiota and predation by protozoa are some of the possible mechanism (Curtis, 2003). Different spatial configuration than conventional flocs and microbiota stratification, such as that found in granular sludge, was proposed to influence the removal of ARGs. However, we observed that systems based on granular sludge provided a comparable removal of both antibiotics and also ARGs than a parallel system based on flocculent activated sludge (**Chapter 3**), although on the lower range of the performance obtained for Dutch WWTPs (**Chapter 2**). A similar or even better removal of both ARGs and antibiotics in granular sludge systems compared to conventional systems was reported elsewhere (Sabri et al., 2020). Noticeably, the removal of *ermB* gene was significantly worse in the treatment based on granular sludge than in the secondary treatment based on flocculent sludge. Even lower removal of *ermB* (-0.08 logs) has been described in another full-scale plant based on granular sludge (Sabri et al., 2020), but no causes have been suggested. Here we hypothesize that such differences might be associated with a lower migration of bacteria containing *ermB* gene (i.e., *Lactobacillales*) towards the sludge granular sludge fraction, as mentioned in **section 6.3.2.**

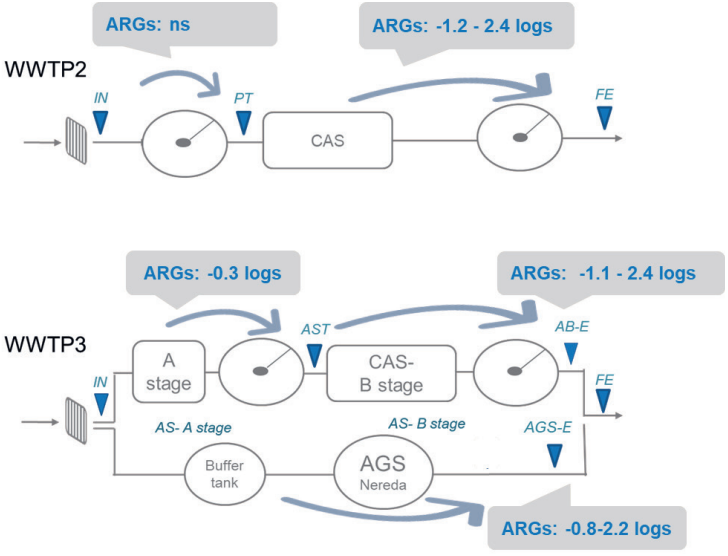


Figure 6.1. Removal of ARGs throughout the varied stages of a wastewater system based on conventional activated sludge (CAS) treatment (WWTP2), AB system (WWTP3 top) and aerobic granular sludge (AGS) Nereda ® (WWTP3 bottom). Legend: ns: no significant reduction. Remaining of abbreviations as in Figure 3.1

6.5. Integrating flow information in ARGs surveillance in wastewater

The recent pandemic of Covid19 (Sars-CoV-2) has highlighted the use of wastewater as an essential and cost-effective tool for surveillance on microbiological agents of fecal origin. In general, the surveillance of water-borne microbiological agents is based on measuring concentrations per unit of sewage (i.e., CFUs or gene copies per mL or 100 mL). However, wastewater is often susceptible to fluctuations in flow caused by water sources other than household use, i.e., rainfall and industrial activity, possibly influencing the measured concentrations of the studied agents. Neglecting to account for such flow variation can bias the results of water-borne pathogens surveillance. Recent works addressing Sars-CoV-2 have tracked in parallel CrAssphage (Green et al., 2020), a bacteriophage virus, associated only with fecal bacteria (Karkman et al., 2019). CrAssphage was used to normalize the concentration of Sars-CoV-2 for samples intercomparison. Possible dilution phenomena could also be inferred by observing a correlated decrease of the CrAssphage and the agent of interest (Sars-CoV-2 or ARGs). With a similar approach, CrAssphage could be of use for the normalization of ARGs data.

Another possibility to account for the flow variations, helping assess accurate fluxes of contaminants, is the use of "loads" (concentration times the total volume processed per unit of time, i.e., day) of the investigated agent. Loads are often used in micropollutants research (Marx et al., 2015). Surprisingly, this concept is not commonly applied in the surveillance of fecal-related microbiological agents. Here, we integrated the use of loads and compared them to concentrations. Assuming a constant discharge of ARGs (i.e., well-established gut resistome in the population), loads (unlike concentrations) of ARGs should not vary despite differences in flow if the faecal matter was the only source for this agent. Thus, we suggest that loads are a better response variable to address the effect of factors affecting the prevalence of infectious agents in wastewater (**Chapter 3**). In line with this hypothesis, it has been recently reported that the use of loads of Sars-CoV-2 instead of concentration was more accurate to predict the number of acute Covid19 infections (Westhaus et al., 2021).

In addition, to achieve the intra and intercomparison of the effects of flow across a single or varied WWTPs, we coined the term Hydraulic Load Factor. The Hydraulic Load Factor is based on the ratio of the flow of the day of sampling regarding the WWTP's basal flow. To calculate the latter, we used the average annual flow instead of the dry weather flow, which was not available for all WWTPs. In **Chapter 3**, we used both concentrations and load (or load

normalized per population equivalent) of ARGs or *E. coli* in the function of the hydraulic load factor to identify the effect of flow variability in the occurrence of both agents. When using concentrations, a dilution effect was observed for *E. coli* but not for the genes. By using loads in the function of the Hydraulic Load Factor, we observed a significant increase of resistance genes with increasing flow, unlike for *E. coli* that was stable. We hypothesized that a fraction of the ARGs arriving at the influent during high flow events might originate from the washout of sewer stocks (biofilms and sediments).

Integrating the flow into the study design was critical to understand the removal efficiency of the wastewater treatment. Some studies in the past years have attempted to unravel some abiotic parameter that correlates with an increase or decrease of genes or resistant bacteria (Korzeniewska and Harnisz, 2018; Novo and Manaia, 2010) but not common effect was observed for the ARGs as a whole. In **Chapter 2** we reported that the increasing flow had a similar effect (reduction) towards all the tested genes. We calculated that the removal of resistance genes is reduced, on average, -0.35 to -0.38 logs for every increase in the average daily flow (**Chapters 3** and **2**, respectively). This amount was even significantly lower (-0.69 to -0.83 logs) for some specific genes (*ermB, qnrS, tetM*). In **Chapter 3** we corroborated the effect of increasing flow as the major cause of variability in the removal of ARGs in a long-term study. In addition, we observed an even stronger detrimental effect for the removal of *E. coli*, being reduced by -0.53 logs for every increase in the average daily flow.

These results grant new perspectives for controlled surveillance of discharged ARGs and bacteria during high flow periods and the intercomparison across sampling campaigns under fluctuating precipitation regimes. They might also apply for WWTPs with seasonal flow variations caused by industrial activity. In addition, our findings bring a new possibility for mitigation strategies. Some suggestions are the addition of equalization tanks or the redesign of the secondary clarifiers for better coping with these flow variations. Our results also highlight the importance of including information about the flow during the sampling campaigns in either the formal analysis or in the metadata. Since different climates and sewer design might result in varying severity of flow's effect, we invite the scientific community to address the effect of flow in the occurrence and removal of ARGs, resistant bacteria and different water-borne pathogens across other locations.

6.6. Transfer of conjugal plasmids in wastewater treatment systems

One of the arguments pointing to WWTPs as sources of antibiotic resistance is the hypothesis that these environments provide unique opportunities for horizontal gene transfer of plasmid-borne ARGs (Manaia et al., 2018). Many different incompatibility (Inc) families are associated with plasmid-borne transmission of ARGs (Rozwandowicz et al., 2018). Yet, broad host range plasmids such as those of the IncP-1 family are the principal vectors used to assess the dissemination of ARGs in complex biological systems, such as activated sludge. In **Chapters 2** and **3**, we observed that broad host range IncP-1 plasmids were indeed prevalent in wastewater (10^5 gene copies mL^{-1}), activated sludge (10^8-10^{10} copies gTS^{-1}) and also in the effluent (~10^4 gene copies mL^{-1}). In fact, a significant increase in the relative abundance of IncP-1 plasmids after treatment was observed. This change was not correlated with the remaining ARGs or MGE studied here, pointing to a non-exclusive association with this specific plasmid family. Although IncP-1 plasmids are often linked with ARGs, they might as well present only resistance to other elements (i.e. metals), harbour only metabolic traits or completely lack known accessory genes (Brown et al., 2013). Even when they initially lack ARGs, IncP-1 are often considered vehicles for antibiotic resistance exchange due to their association with several MGEs, causing a high gene acquisition and loss (Shintani et al., 2020). This exchange might occur while co-existing with other plasmids in the same cell. Conjugal transfer of IncP-1 to cells already containing another plasmid(s) is common, as also observed by the successful transfer towards wild-type multi-plasmid *E. coli* in **Chapter 4.**

Conjugation has been studied for decades. Still, there is limited information on the real transfer rates of plasmids in wastewater and activated sludge systems. A considerable portion of the knowledge about conjugation (pointing to substantial transfer rates) are derived from *in vitro* studies conducted under favourable conditions (optimal cell densities, temperature and nutrients) facilitating plasmid transfer. In **Chapter 4** we evaluated (*in vitro*) how this transfer differed if performed under optimal laboratory conditions and environmental-related temperatures and nutrients. We observed that the conjugal transfer remained high (10^{-1} to 10^{-2} T/D) when moving from optimal (37ºC) to environmental (25, 15, 9ºC) temperatures, but was significantly hindered by low nutrient availability such as in synthetic wastewater and especially, soil extract, in comparison with the rich (LB) nutrient media.

In situ assessment of conjugal transfer within complex biological systems such as wastewater and activated sludge or soil continues to be challenging (Sørensen et al., 2005). In

the last two decades, new opportunities emerged with fluorescently labelled plasmids and strains and the use of culture-independent detection methods such as flow cytometry. However, up to now, these methods have been used along with an *in vitro* cultivation step (Klümper et al., 2015; Li et al., 2018a) in order to obtain the maximum transconjugant numbers. Recently, Jong et al. (2020) combined fluorescently labelled plasmids and direct detection in microcosms, yet, the lack of fluorescently labelled donor (meaning donor and transconjugants showed the same fluorescent signal) prevented the quantification of true transconjugants. This thesis (**Chapter 5**), using a double reporter system (donors and plasmid presenting different fluorescence (Musovic et al., 2010)), demonstrated that a direct recovery and quantification of IncP-1 transconjugants after 24h mating in sludge microcosms is possible. However, we also found that the donors persisted under certain mating conditions (i.e., low temperatures or anaerobic conditions), accounting for most recovered cells, which hindered the quantification of transconjugants. In general, the low transconjugant numbers also prevented its identification by direct sequencing after cells sorting. Furthermore, the use of this approach might not be suitable for the investigation of conjugal transfer of narrow host range plasmids (i.e., IncI) in sludge systems, where the putative recipient community (Enterobacteriaceae) is relatively small (~ 10^3 CFUs mL^{-1} of sludge).

6.7. Concluding remarks.

This work provides evidence that conventional WWTPs are fundamental end of the pipe solutions to reduce the emissions of anthropogenic-related antibiotics and resistant determinants towards surface water environments. Possible factors hindering their removal capacity (high flow, effluent suspended solids) are identified, and their effect analysed quantitatively. The first steps towards a more realistic evaluation of the potential transfer of conjugal plasmids within wastewater treatment are also performed.

In a global context, the results presented here may help to reinforce the urgency for upgrading wastewater sanitation towards secondary treatment based on activated sludge (flocculent or granular). Moreover, this thesis highlights the relevance of integrating flow dynamics into wastewater surveillance, providing more accurate quantification of the biological targets. Accounting for the (predominant) effect of flow may also help to unravel the influence of alternative treatment parameters or abiotic factors on removing resistance determinants.

Although further efforts in understanding the dynamics of antibiotic resistance in sewers and WWTP are necessary, it is also essential to realize that most part of the global population

still does not have access to adequate sanitation. Constant raw sewage discharges (caused by lack of available basic wastewater treatment) contribute to enlarge the environmental resistome and pose a risk to the public health and to the progress against antibiotic resistance in particular. A lesson learnt from the current Covid19 pandemic is that a common international strategy against health threats in our best approach and thus, joint efforts must be applied to ensure universal access to sanitation and wastewater treatment.

6.8. Future recommendations.

In view of the results obtained in this thesis and a review of the current literature, knowledge gaps and follow up research topics are suggested.

6.7.1. Microbial Risk Assessment with available data on antibiotic resistance in The Netherlands.

In the last years, a remarkable effort has been made to increase our knowledge of antibiotics and antibiotic resistance in wastewater and environmental systems worldwide. In parallel to this thesis, other studies have also been conducted in The Netherlands, completing the information about the occurrence of antibiotics (Sabri et al., 2018), resistant determinants and antibiotic resistant bacteria in Dutch sewage and surface waters (Paulus et al., 2020; Sabri et al., 2020, 2018; Verburg et al., 2019). It is about time to integrate these results into risk assessment models to predict the actual risk of human or animal exposure to wastewater and water-borne antibiotic resistance. The outcome of such an assessment may be of use for the relevant authorities to decide whether new discharge limitations based on antibiotic resistance indicators are needed.

The leading challenge when evaluating microbial risks is translating the risk of exposure to resistant bacteria into risks for human health and predicting the infection rate. Data on resistant bacteria rather than ARGs would be more accurate to estimate the risk of infection. Yet, information on ARGs concentration might also be of use to estimate the probability of contact with antibiotic-resistant microorganisms. Current investigations in extracellular and intracellular DNA fractions (Calderón-Franco et al., 2021; Calderon-Franco et al., in preparation) have proved that the latter is the predominant form of detected DNA in wastewater. Thus, it can be assumed that the detected ARGs levels correspond to living microorganisms. However, a correction factor accounting for possible multicopy genes might be necessary to estimate the actual number of resistant bacteria.

6.7.2. Further abatement of priority antibiotics and ARGs.

Despite the positive trends observed in the reduction of antibiotics and ARGs during wastewater treatment, both agents continue to be discharged to surface waters. In the context of increasing water scarcity, wastewater effluent is also regarded as a complementary source for irrigation water (known as reclaimed water). Yet, once again, the chemical and microbiological quality of effluents needs to be further improved to be suitable for consideration for water reclamation and irrigation.

New initiatives to reduce pharmaceuticals and priority compounds in wastewater, including antibiotic residues, are currently ongoing in The Netherlands ("ketenaanpak medicijnresten"). Yet, it remains crucial to acknowledge that different technologies have a dissimilar impact on the diverse antimicrobial compounds (Sabri, 2020).

In the European framework, additional efforts tackling antibiotics should be directed towards the elimination of ciprofloxacin and azithromycin. Those are the substances with the highest detected concentration in the effluent. More importantly, these concentrations often exceed the PNEC-MIC level (0.06 and 0.25 μg $L^{-1,}$ for ciprofloxacin and azithromycin, respectively), indicating the potential for selecting antibiotic-resistant bacteria (**Chapter 3, 6**, Carvalho and Santos, 2016; Rodriguez-Mozaz et al., 2020). Given their effluent levels in Europe above the PNEC-ENV (0,08 μg L^{-1}), the cephalosporin cefalexin (not measured in our study) has also been appointed as a priority antibiotic (Rodriguez-Mozaz et al., 2020). Some promising techniques for the complementary elimination of antibiotics are adsorption by activated carbon and oxidation by ozone or other advanced oxidation techniques (Rizzo et al., 2020). Possible challenges are the (1) generation of by-products that might be more hazardous than the original compounds and (2) the combination of pharmaceuticals removal with the removal of resistant microorganisms, as the latter are presumably not affected by the same elimination pathways.

Aside from the possible implications for human health (pending the risk assessment evaluation as previously mentioned), it is already demonstrated that In the Netherlands, effluent discharges of ARGs and antibiotic resistant bacteria are increasing the basal resistome in the receiving freshwaters (Sabri et al., 2018; Verburg et al., 2019). Therefore, further reduction of resistant determinants by water treatment is needed. However, such an extra removal seems more challenging than in the case of antibiotics. Different advanced treatments (i.e. chlorination, UV disinfection, ozonation) have been already implemented after the secondary treatments in other countries (Di Cesare et al., 2016; Rodriguez-Mozaz et al., 2020; Wen et al., 2016). Yet,

whether these extra treatments stages deliver a significant improvement is still under debate. Comparable removal rates to the ones observed in this thesis (with WWTPs based only on secondary treatment) were observed for studies including WWTPs with different advanced treatment (Di Cesare et al., 2016; Wen et al., 2016). In addition, promising initial reductions might be hampered by the selection of resistant bacteria (Fiorentino et al., 2015), photoreactivation and bacterial regrowth (Fiorentino et al., 2015; Sousa et al., 2017). Physical separation technologies (like membrane-based processes) are an alternative to bypass those effects, although likely not an affordable option in all situations due to their elevated economic costs and energy consumption (Kehrein et al., 2020). Indeed, providing low-cost alternatives for widespread use in low or middle-income countries (i.e. adsorption by biochar) remains fundamental as well (Calderón-Franco et al., 2021a).

6.7.3. Unravelling the effect of flow and seasonal variations in other climatic regions.

Here we conclude that flow but not seasonal temperature hindered the removal of ARGs. Evidently, the particulars in precipitation and temperature regimes (along with catchment infrastructures) in other locations might compromise the accuracy of our predictions. Therefore, validating the observations provided in other sites would be fundamental in the quest to unravel the factors affecting the removal of ARGs throughout wastewater treatment.

If several studies are performed, a model integrating all the information might be helpful for global surveillance in antibiotic resistance and for local authorities to decide if additional mitigation measures must be taken under certain climatic conditions.

6.7.4. Nutrient effect on conjugal transfer

In **Chapter 4** we observed that conjugal transfer is nutrient-dependent. Our preliminary work highlights the possible importance of nutrients available in natural and engineered ecosystems. Surprisingly, very little is known about the role of specific essential nutrients (nitrogen-based, phosphorus/phosphate-based or specific cations) on the conjugal transfer. This topic deserves further investigation for several reasons. First, because it will help to provide a more accurate transfer rate in real ecosystems. Second, because it might comprise a route for preventing the spread of plasmid-borne antibiotic resistance. The actual restrictions for nitrogen and phosphorus emissions by Dutch WWTPs, especially to those discharging in the North Sea catchment, might already mitigate plasmid-mediated resistance propagation. Yet complementary research is necessary to prove this hypothesis. Possible applications of the

results might be implementing similar restrictions to other WWTPs with less constrained discharge requirements, both in the Netherlands but also in other parts of the globe.

Experiments could focus on understanding the impact of nutrients on the different species that might be relevant to the spread of antibiotic resistance in wastewater environments. Likely, conjugal transfer in common water-borne pathogens would be less affected than gut microbiota bacteria by limited nutrient conditions, as Goodman et al. (1993) observed for *Vibrio* spp and *E. coli*. Researching both scenarios is necessary. Enterobacteria (*E. coli*, *Klebsiella* spp, *Salmonella* spp) will likely harbour the most crucial ARGs (related to last resource antibiotics). Other nosocomial agents better adapted for environmental conditions such as A*eromonas spp*., *Pseudomonas aeruginosa* are suggested as relevant agents to be studied for this proposal. The latter may persist longer in the environment and thus encounter more opportunities for the conjugal transfer of ARGs.

6.7.5. *Optimization of* in situ *monitoring of conjugal transfer and identification of key vectors.*

In **Chapter 5** we proved that direct detection and quantification of transconjugants from microcosms experiments in combination with flow cytometry was possible. However, the low number of detected transconjugants hindered the detection of plasmid transfer beyond the optimal conditions (aerobic and 30ºC). Even at optimal conditions, cell sorting and further identification of the transconjugants were not possible. Thus, there is still room for the optimization of the experimental setup. Some ideas are for follow up research in this area are suggested:

(1) Improve cell detachment. An over-representation of the planktonic fraction of the bacterial community in the sludge rather than its core (bacteria within the flocs) might have biased the current results. Preliminary cell detachment with the help of a surfactant before the sonication step, as in Jong et al. (2020), is suggested.

(2) Longer mating periods than the ones used in our experiments (24h) will most likely improve the number of detected transconjugants. However, part of these newly detected transconjugants might originate from clonal expansion. Thus, this approach might not be indicated if transfer rates need to be measured. In the end, transfer rates are indeed crucial to assess the risk of transmission, but plasmid maintenance and persistence in the community (even if it is mainly driven by clonal expansion) also deserve attention.

(3) Ultimately, pre-conditioning of donors to similar (low) nutrients conditions (as original occurring in the real environments) before mating would be desirable, although it might lead once again to a low amount of recovered transconjugants.

6.9. References

Ali, M., Wang, Z., Salam, K.W., Hari, A.R., Pronk, M., Van Loosdrecht, M.C.M., Saikaly, P.E., 2019. Importance of species sorting and immigration on the bacterial assembly of different-sized aggregates in a full-scale Aerobic granular sludge plant. Environ. Sci. Technol. 53, 8291–8301. https://doi.org/10.1021/acs.est.8b07303

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ. Int. 86, 140–149. https://doi.org/10.1016/j.envint.2015.10.015

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439

Blaak, H., Lynch, G., Italiaander, R., Hamidjaja, R.A., Schets, F.M., de Roda Husman, A.M., 2015. Multidrug-Resistant and Extended Spectrum Beta-Lactamase-Producing Escherichia coli in Dutch Surface Water and Wastewater. PLoS One 10, e0127752. https://doi.org/10.1371/journal.pone.0127752

Brown, C.J., Sen, D., Yano, H., Bauer, M.L., Rogers, L.M., Van der Auwera, G.A., Top, E.M., 2013. Diverse broad-host-range plasmids from freshwater carry few accessory genes. Appl. Environ. Microbiol. 79, 7684–7695. https://doi.org/10.1128/AEM.02252-13

Calderón-Franco, D., Apoorva, S., Medema, G., van Loosdrecht, M.C.M., Weissbrodt, D.G., 2021a. Upgrading residues from wastewater and drinking water treatment plants as low-cost adsorbents to remove extracellular DNA and microorganisms carrying antibiotic resistance genes from treated effluents. Sci. Total Environ. 778, 146364. https://doi.org/10.1016/j.scitotenv.2021.146364

Calderón-Franco, D., van Loosdrecht, M.C.M., Abeel, T., Weissbrodt, D.G., 2021b. Free-floating extracellular DNA: Systematic profiling of mobile genetic elements and antibiotic resistance from wastewater. Water Res. 189. https://doi.org/10.1016/j.watres.2020.116592

Carvalho, I.T., Santos, L., 2016. Antibiotics in the aquatic environments: A review of the European scenario. Environ. Int. 94, 736–757. https://doi.org/10.1016/j.envint.2016.06.025

Curtis, T., 2003. Bacterial pathogen removal in wastewater treatment plants, Handbook of Water and Wastewater Microbiology. Elsevier. https://doi.org/10.1016/B978-012470100-7/50031-5

Di Cesare, A., Eckert, E.M., D'Urso, S., Bertoni, R., Gillan, D.C., Wattiez, R., Corno, G., 2016. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. Water Res. 94, 208–214. https://doi.org/10.1016/j.watres.2016.02.049

ECDC, 2018. Surveillance of antimicrobial resistance in Europe 317. https://doi.org/10.2900-22212

ESAC-Net, E., 2018. Antimicrobial consumption in the EU/EEA, annual epidemiological report for 2018. Ecdc 1–24.

EC TGD, 2003. European commission Technical Guidance Document in Support of Commission Directive 93//67/EEC on Risk Assessment for New Notified Substances and Commission Regulation (EC) No. 1488/94 on Risk Assessment for Existing Substance. Part II,, 100–103

Fiorentino, A., Ferro, G., Alferez, M.C., Polo-López, M.I., Fernández-Ibañez, P., Rizzo, L., 2015. Inactivation and regrowth of multidrug resistant bacteria in urban wastewater after disinfection by solar-driven

and chlorination processes. J. Photochem. Photobiol. B Biol. 148, 43–50. https://doi.org/10.1016/j.jphotobiol.2015.03.029

Gracia-Lor, E., Sancho, J. V., Serrano, R., Hernández, F., 2012. Occurrence and removal of pharmaceuticals in wastewater treatment plants at the Spanish Mediterranean area of Valencia. Chemosphere 87, 453–462. https://doi.org/10.1016/j.chemosphere.2011.12.025

Green, H., Wilder, M., Collins, M., Fenty, A., Gentile, K., Kmush, B.L., Zeng, T., Middleton, F.A., Larsen, D.A., 2020. Quantification of SARS-CoV-2 and cross-assembly phage (crAssphage) from wastewater to monitor coronavirus transmission within communities. medRxiv 2, 1–18. https://doi.org/10.1101/2020.05.21.20109181

Hendriksen, R.S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P.T.L.C., Vogt, J.K., Leekitcharoenphon, P., van de Schans, M.G.M., Zuidema, T., de Roda Husman, A.M., Rasmussen, S., Petersen, B., Bego, A., Rees, C., Cassar, S., Coventry, K., Collignon, P., Allerberger, F., Rahube, T.O., Oliveira, G., Ivanov, I., Vuthy, Y., Sopheak, T., Yost, C.K., Ke, C., Zheng, H., Baisheng, L., Jiao, X., Donado-Godoy, P., Coulibaly, K.J., Jergović, M., Hrenovic, J., Karpíšková, R., Villacis, J.E., Legesse, M., Eguale, T., Heikinheimo, A., Malania, L., Nitsche, A., Brinkmann, A., Saba, C.K.S., Kocsis, B., Solymosi, N., Thorsteinsdottir, T.R., Hatha, A.M., Alebouyeh, M., Morris, D., Cormican, M., O'Connor, L., Moran-Gilad, J., Alba, P., Battisti, A., Shakenova, Z., Kiiyukia, C., Ng'eno, E., Raka, L., Avsejenko, J., Bērziņš, A., Bartkevics, V., Penny, C., Rajandas, H., Parimannan, S., Haber, M.V., Pal, P., Jeunen, G.J., Gemmell, N., Fashae, K., Holmstad, R., Hasan, R., Shakoor, S., Rojas, M.L.Z., Wasyl, D., Bosevska, G., Kochubovski, M., Radu, C., Gassama, A., Radosavljevic, V., Wuertz, S., Zuniga-Montanez, R., Tay, M.Y.F., Gavačová, D., Pastuchova, K., Truska, P., Trkov, M., Esterhuyse, K., Keddy, K., Cerdà-Cuéllar, M., Pathirage, S., Norrgren, L., Örn, S., Larsson, D.G.J., Heijden, T. Van der, Kumburu, H.H., Sanneh, B., Bidjada, P., Njanpop-Lafourcade, B.M., Nikiema-Pessinaba, S.C., Levent, B., Meschke, J.S., Beck, N.K., Van, C.D., Phuc, N. Do, Tran, D.M.N., Kwenda, G., Tabo, D. adjim, Wester, A.L., Cuadros-Orellana, S., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J.R.M., Aidara-Kane, A., Pamp, S.J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M.P., Vigre, H., Petersen, T.N., Aarestrup, F.M., 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat. Commun. 10. https://doi.org/10.1038/s41467-019-08853-3

Henze, M., van Loosdrecht, M.C.M., Ekama, G.A., Brdjanovic, D., 2005. Biological wastewater treatment, Chemical Engineering.

Jong, M.C., Harwood, C.R., Blackburn, A., Snape, J.R., Graham, D.W., 2020. Impact of Redox Conditions on Antibiotic Resistance Conjugative Gene Transfer Frequency and Plasmid Fate in Wastewater Ecosystems. Environ. Sci. Technol. 54, 14984–14993. https://doi.org/10.1021/acs.est.0c03714

Jutkina, J., Rutgersson, C., Flach, C.F., Joakim Larsson, D.G., 2016. An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. Sci. Total Environ. 548–549, 131–138. https://doi.org/10.1016/j.scitotenv.2016.01.044

Karkman, A., Pärnänen, K., Larsson, D.G.J., 2019. Fecal pollution can explain antibiotic resistance gene abundances in anthropogenically impacted environments. Nat. Commun. 10, 1–8. https://doi.org/10.1038/s41467-018-07992-3

Kehrein, P., Van Loosdrecht, M., Osseweijer, P., Garfí, M., Dewulf, J., Posada, J., 2020. A critical review of resource recovery from municipal wastewater treatment plants-market supply potentials, technologies and bottlenecks. Environ. Sci. Water Res. Technol. 6, 877–910. https://doi.org/10.1039/c9ew00905a

Keller, V.D.J., Williams, R.J., Lofthouse, C., Johnson, A.C., 2014. Worldwide estimation of river concentrations of any chemical originating from sewage-treatment plants using dilution factors. Environ. Toxicol. Chem. 33, 447–452. https://doi.org/10.1002/etc.2441

Klümper, U., Smets, B.F., Dechesne, A., 2015. Permissiveness of soil microbial communities towards broad host range plasmids.

Korzeniewska, E., Harnisz, M., 2018. Relationship between modification of activated sludge wastewater treatment and changes in antibiotic resistance of bacteria. Sci. Total Environ. 639, 304–315. https://doi.org/10.1016/j.scitotenv.2018.05.165

LaPara, T.M., Burch, T.R., McNamara, P.J., Tan, D.T., Yan, M., Eichmiller, J.J., 2011. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. Environ. Sci. Technol. 45, 9543–9549. https://doi.org/10.1021/es202775r

LaPara, T.M., Madson, M., Borchardt, S., Lang, K.S., Johnson, T.J., 2015. Multiple Discharges of Treated Municipal Wastewater Have a Small Effect on the Quantities of Numerous Antibiotic Resistance Determinants in the Upper Mississippi River. Environ. Sci. Technol. 49, 11509–11515. https://doi.org/10.1021/acs.est.5b02803

Lee, J., Ju, F., Maile-moskowitz, A., Beck, K., Maccagnan, A., Mcardell, C.S., Dal, M., Fenicia, F., Vikesland, P.J., Pruden, A., Stamm, C., Bürgmann, H., 2021. Unraveling the riverine antibiotic resistome : The downstream fate of anthropogenic inputs. Water Res. 197, 117050. https://doi.org/10.1016/j.watres.2021.117050

Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Gaze, W.H., 2015. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. Environ. Int. 82, 92–100. https://doi.org/10.1016/j.envint.2015.02.013

Li, L., Dechesne, A., He, Z., Madsen, J.S., Nesme, J., Sørensen, S.J., Smets, B.F., 2018. Estimating the Transfer Range of Plasmids Encoding Antimicrobial Resistance in a Wastewater Treatment Plant Microbial Community. Environ. Sci. Technol. Lett. 5, 260–265. https://doi.org/10.1021/acs.estlett.8b00105

Li, L., Nesme, J., Quintela-Baluja, M., Balboa, S., Hashsham, S., Williams, M.R., Yu, Z., Sørensen, S.J., Graham, D.W., Romalde, J.L., Dechesne, A., Smets, B.F., 2021. Extended-Spectrum β-Lactamase and Carbapenemase Genes are Substantially and Sequentially Reduced during Conveyance and Treatment of Urban Sewage. Environ. Sci. Technol. 55, 5939–5949. https://doi.org/10.1021/acs.est.0c08548

Ma, Y., Wilson, C.A., Novak, J.T., Riffat, R., Aynur, S., Murthy, S., Pruden, A., 2011. Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and class i integrons. Environ. Sci. Technol. 45, 7855–7861. https://doi.org/10.1021/es200827t

Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I.C., Zammit, I., Kampouris, I., Vaz-Moreira, I., Nunes, O.C., 2018. Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environ. Int. 115, 312–324. https://doi.org/10.1016/j.envint.2018.03.044

Marx, C., Mühlbauer, V., Schubert, S., Oertel, R., Ahnert, M., Krebs, P., Kuehn, V., 2015. Representative input load of antibiotics to WWTPs: Predictive accuracy and determination of a required sampling quantity. Water Res. 76, 19–32. https://doi.org/10.1016/j.watres.2015.02.049

Merlin, C., 2020. Reducing the Consumption of Antibiotics: Would That Be Enough to Slow Down the Dissemination of Resistances in the Downstream Environment? Front. Microbiol. 11, 1–4. https://doi.org/10.3389/fmicb.2020.00033

Musovic, S., Dechesne, A., Sørensen, J., Smets, B.F., 2010. Novel Assay To Assess Permissiveness of a Soil Microbial Community toward Receipt of Mobile Genetic Elements. Appl. Environ. Microbiol. 76, 4813–4818. https://doi.org/10.1128/AEM.02713-09

Novo, A., Manaia, C.M., 2010. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. Appl. Microbiol. Biotechnol. 87, 1157–1166. https://doi.org/10.1007/s00253-010-2583-6

Pärnänen, K.M.M., Narciso-Da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V. Della, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5. https://doi.org/10.1126/sciadv.aau9124

Paulus, G.K., Hornstra, L.M., Medema, G., 2020. International tempo-spatial study of antibiotic resistance genes across the Rhine river using newly developed multiplex qPCR assays. Sci. Total Environ. 706, 135733. https://doi.org/10.1016/j.scitotenv.2019.135733

Quintela-Baluja, M., Abouelnaga, M., Romalde, J., Su, J.Q., Yu, Y., Gomez-Lopez, M., Smets, B., Zhu, Y.G., Graham, D.W., 2019. Spatial ecology of a wastewater network defines the antibiotic resistance genes in downstream receiving waters. Water Res. 162, 347–357. https://doi.org/10.1016/j.watres.2019.06.075

Rafraf, I.D., Lekunberri, I., Sànchez-Melsió, A., Aouni, M., Borrego, C.M., Balcázar, J.L., 2016. Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. Environ. Pollut. 219, 353–358. https://doi.org/10.1016/j.envpol.2016.10.062

Rizzo, L., Gernjak, W., Krzeminski, P., Malato, S., Mcardell, C.S., Antonio, J., Perez, S., Schaar, H., Fatta-kassinos, D., 2020. Science of the Total Environment Best available technologies and treatment trains to address current challenges in urban wastewater reuse for irrigation of crops in EU countries. Sci. Total Environ. 710, 136312. https://doi.org/10.1016/j.scitotenv.2019.136312

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242. https://doi.org/10.1016/j.watres.2014.11.021

Rodriguez-Mozaz, S., Vaz-Moreira, I., Varela Della Giustina, S., Llorca, M., Barceló, D., Schubert, S., Berendonk, T.U., Michael-Kordatou, I., Fatta-Kassinos, D., Martinez, J.L., Elpers, C., Henriques, I., Jaeger, T., Schwartz, T., Paulshus, E., O'Sullivan, K., Pärnänen, K.M.M., Virta, M., Do, T.T., Walsh, F., Manaia, C.M., 2020. Antibiotic residues in final effluents of European wastewater treatment plants and their impact on the aquatic environment. Environ. Int. 140, 105733. https://doi.org/10.1016/j.envint.2020.105733

Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Guerra, B., Mevius, D.J., Hordijk, J., 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J. Antimicrob. Chemother. 73, 1121–1137. https://doi.org/10.1093/jac/dkx488

Sabri, N. 'Azyyati binti, 2020. Antibiotics and antibiotic resistance genes in wastewater: occurrence and removal technologies. PhD Thesis. Wageningen University. https://doi.org/10.18174/524759

Sabri, N.A., Schmitt, H., Van der Zaan, B., Gerritsen, H., Zuidema, T., Rijnaarts, H.H.., Langenhoff, A.A.M., 2018. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. J. Environ. Chem. Eng. https://doi.org/10.1016/j.jece.2018.03.004

Sabri, N.A., van Holst, S., Schmitt, H., van der Zaan, B.M., Gerritsen, H.W., Rijnaarts, H.H.M., Langenhoff, A.A.M., 2020. Fate of antibiotics and antibiotic resistance genes during conventional and additional treatment technologies in wastewater treatment plants. Sci. Total Environ. 741, 140199. https://doi.org/10.1016/j.scitotenv.2020.140199

Schmitt, H., Stoob, K., Hamscher, G., Smit, E., Seinen, W., 2006. Tetracyclines and tetracycline resistance in agricultural soils: Microcosm and field studies. Microb. Ecol. 51, 267–276. https://doi.org/10.1007/s00248-006-9035-y

Scornec, H., Bellanger, X., Guilloteau, H., Groshenry, G., Merlin, C., 2017. Inducibility of Tn916 conjugative transfer in Enterococcus faecalis by subinhibitory concentrations of ribosome-targeting antibiotics. J. Antimicrob. Chemother. 72, 2722–2728. https://doi.org/10.1093/jac/dkx202

Shintani, M., Nour, E., Elsayed, T., Blau, K., Wall, I., Jechalke, S., Spröer, C., Bunk, B., Overmann, J., Smalla, K., 2020. Plant Species-Dependent Increased Abundance and Diversity of IncP-1 Plasmids in the Rhizosphere: New Insights Into Their Role and Ecology. Front. Microbiol. 11, 1–11. https://doi.org/10.3389/fmicb.2020.590776

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., 2005. Studying plasmid horizontal transfer in situ: a critical review. Nat. Rev. Microbiol. 3, 700–710. https://doi.org/10.1038/nrmicro1232

Sousa, J.M., Macedo, G., Pedrosa, M., Becerra-Castro, C., Castro-Silva, S., Pereira, M.F.R., Silva, A.M.T., Nunes, O.C., Manaia, C.M., 2017. Ozonation and UV254nm radiation for the removal of microorganisms and antibiotic resistance genes from urban wastewater. J. Hazard. Mater. 323, 434–441. https://doi.org/10.1016/j.jhazmat.2016.03.096

van Krimpen, P.C., van Bennekom, W.P., Bult, A., 1987. Penicillins and cephalosporins. Physicochemical properties and analysis in pharmaceutical and biological matrices. Pharm. Weekbl. 9, 1–23. https://doi.org/10.1007/BF01956487

Verburg, I., García-Cobos, S., Leal, L.H., Waar, K., Friedrich, A.W., Schmitt, H., 2019. Abundance and antimicrobial resistance of three bacterial species along a complete wastewater pathway. Microorganisms 7. https://doi.org/10.3390/microorganisms7090312

Wen, Q., Yang, L., Duan, R., Chen, Z., 2016. Monitoring and evaluation of antibiotic resistance genes in four municipal wastewater treatment plants in Harbin, Northeast China. Environ. Pollut. 212, 34–40. https://doi.org/10.1016/j.envpol.2016.01.043

Westhaus, S., Weber, F.A., Schiwy, S., Linnemann, V., Brinkmann, M., Widera, M., Greve, C., Janke, A., Hollert, H., Wintgens, T., Ciesek, S., 2021. Detection of SARS-CoV-2 in raw and treated wastewater in Germany – Suitability for COVID-19 surveillance and potential transmission risks. Sci. Total Environ. 751, 141750. https://doi.org/10.1016/j.scitotenv.2020.141750

WHO, 2017. Global framework for development & stewardship to combat antimicrobial resistance - draft roadmap. OIE World Organ. Anim. Heal. 08, 29.

Yang, Y., Li, B., Zou, S., Fang, H.H.P., Zhang, T., 2014. Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. Water Res. 62, 97–106. https://doi.org/10.1016/j.watres.2014.05.019

Epilogue

Worldwide, 2.2 billion people still lack access to safe drinking water.

More than half of the global population does not have access to safe sanitation.

Three billion people do not have access to handwashing facilities with soap.

Still, 673 million people practice open defecation.

WASH initiative facts

(https://www.unicef.org/wash)

Acknowledgements

You probably already know that the last 5 years have been an emotional and thrilling rollercoaster for me. I have discovered, learnt, laughed and cried… a lot. The last pages of this thesis are dedicated to all of you who have walked alongside me through this long and bumpy path. I wouldn't have made it without all of you. I hope my next lines will reflect your influence and contribution to this thesis and to my PhD life.

First of all, thanks to all my four supervisors. **Lucía**, you and **Sanne** gave me the opportunity to come to Wetsus in the first place for my MSc Internship. **Lucía**, you trusted me and offered me to continue with a PhD, even when I was not sure I was made for it. I will always be grateful for that. Within the last five years, we have had many ups and downs. Yet, I know you cared for me and you looked out for all the possible resources to make our research possible. **Heike**, thank you for all the knowledge you brought into the AMR group. I have enjoyed our discussions and brainstorming moments; I have definitively learnt a lot. I thank you for your patience, especially while explaining me the statistical concepts over and over again. I am also grateful you encouraged us to learn R (source of equal parts of fulfilment and frustrations). **David W,** thank you a lot for the great personal and scientific spirit you have brought into this project. I know you put all your time and soul in everything you do. I admired that you permanently found new questions to ask in every round of corrections. Beyond your academic help, I would like to thank you for your understanding, your support and making me feel part of the TU Delft group, even though I was far away. **MArk**, you are always ¨benieuwd¨ and you transmit such moto to all your students, encouraging them to go beyond the obvious and mainstream and follow curiosity-driven research. You were often busy and away in all corners of the world, yet you always managed to answering all my queries and giving me the precise advice or the contact person that I needed. I will miss your e mails at all possible times of the day (and night). I hope your correspondence will be easier now with just one Rebeca to message to 😊.

I would never have been able to do this thesis, if earlier on **Manuel and Platero** wouldn't have given me the opportunity to work in their lab at UGR and improve my practical skills. Thank you, **Platero,** for all your patience and for transmitting me your passion for scientific research.

There are many others that have contributed to the work in this thesis with their time, knowledge and discussions. I thank the **members of the Source Separated Sanitation** teams for their interest and questions during our theme meetings. I especially thank Bonnie, who always had encouraging words for my research and was a vital bridge in my communication with the wastewater treatment experts. I am grateful to **Leon** for allowing me to sneak in his meetings with Thomas to ask some ¨quick¨ questions. I would never be able to thank enough **Paul Weij** for an entire year of collecting samples for me and showing so much interest in my project.

I want to thank the colleagues at WBVR, **Dik**, **Daniela**, **Apostolos**, **Mike**, and **Arie**. Although my time at WBVR was short and hectic, you made me feel at home. I am grateful for all you taught me and for your patience when the experiments did not work as planned. I know I still owe you a basket with goodbye muffins. Hopefully, I can deliver them with this thesis.

I also would like to thank professor **Sørensen,** and colleagues from KU **Joseph, Anette** and **Rafa** that made our short stay possible and taught us so much. Special mention to **Asmus,** for his invaluable 24/7 support with the flow cytometry experiments. To **Elise**, **Rosangela**, **Shashank**, thank you for sparking the little spare time we had in that fantastic city.

Thank you to my REPARES colleagues, especially to **David Calderón** and **Dana**. **David**, gracias por hacerme sentir un poquito parte de TUDelft y por estar siempre ahí (en WhatsApp) para lo que necesitase. **Dana**, thanks for your kindness and hard work within REPARES. I have truly enjoyed working with you

All my work at Wetsus wouldn't be possible without the support team at wetsus. First of all, **Gerrit**. Thanks A LOT for being so kind and for your incredible work, providing a perfect organization for the supplies. We all give you a hard time with our ¨urgent¨ request and you are always there to manage no matter what. **Gerben**, always inspiring seeing someone so kind and passionate about his work. I have enjoyed your new occurrence for the "sandwich of the month" and your creative ideas for our thematic borrels. Many thanks as well to the analytical team (**Ton, Jan-Willem, Lisette, Mieke and Marianne, Erwin**). My antibiotic analysis in sludge samples were a challenge, but you managed to make it possible. Of course, a special mention to the microlab team: **Sanne, Bianca** and **Aga** at first, and then growing with the addition of **Inez, Alicia, Bert** and **Pieter**. I know you all will miss me in the microlab meetings (it is quiet now, isn´t it?). I have special thank words to **Bianca**, thank you for your help during the sampling campaign and for your joyful spirit, and a complaint to **Pieter**: why didn't you come to our microlab lives earlier? You are an amazing scientist and a great asset to the microlovers community. **Aga,** you were my first contact in the Wetsus lab back in the old building. Since then, you have been invaluable support both with my struggles in the lab but also in my personal life. You were always there to listen and to give advice. I thank as well to **Caroline** and **Cristina** for enriching the microlovers community at Wetsus with their expertise and questions.

Wetsus is an incredible place to work mainly because of the human quality that it harbours. I have always been lucky with the colleagues I have found along my academic path, and in Wetsus, this has not been an exception.

I first would like to thank to my numerous office mates: To the first generation in the office 1.10C: **Jouke**, showing what hyper focus looks like. **Pau**, thanks for integrating me into the Spanish community. **Sam**, you are such a unique character… I have definitively enjoyed your strong personality and your crazy ideas and stories. **Prashanth**, thank you for being so curious and excited to learn new things. I will always remember our balloons session. Special mention to my core, my latino community: **Mariana** and **Héctor**. Para mi habéis sido el alma de la oficina, y os he echado de menos cada momento desde que os fuisteis. Ha sido tan bonito teneros cerca, conversar, los bailes, las fiestas. Vuestro apoyo ha sido imprescindible en los vaivenes de esta tesis. Gracias por ayudarme a mantener mi español a flote y por hacerme reír con las múltiples desconocidas acepciones del español ¨mejicano¨. **Héctor**, gracias también por ser mi pareja de baile. Los viernes de salsa contigo han sido el soplo de aire que necesitaba (aunque se nos olviden los pasos todo el rato jaja). To the second generation (**Emanuel**, **Rose, Marco, Diego, Gosia, Kaustub, Emad and Barbara**), unfortunately I met you on my busiest moment, and thus I wasn´t much around the office. Yet, thank you for the good moments, the chats and

the brainstorming moments for Sinterklaas poems. To my third round of officemates in the upper floor, at 2.09 A: it was brief but intense. Thank you, **Sandra** and **Casper**, for our shared discussions about Wetsus, mental health and struggles.

To **Rik**, **Natascha**, **Fabian**, **Jaap**, with whom I shared many moments when I was a student and later on as a PhD. **Natascha**, I miss your spontaneous hugs.

To my friends of the first WaterSEED generation: **Tania**, I am glad we shared our PhD representative moments, you for your inspiring strength in defending your thoughts. **Raquelita** you are such a unique, kind and helpful person. You have an incredible determination to overcome all the curveballs the PhD has thrown to you, a true inspiration for everyone in the lab. I will miss our chitchats in Spanish and being mistaken with you all the time. **Paulina**, chica, vaya viaje nos metiste. Gracias por enseñarnos las maravillas de Méjico. P.D. La próxima vez pon 3 días más de playa, porfa. **Maarten**, solo bebidas, por favor. Thank you for being so authentic. I really enjoy your company, our game nights and shopping days.

To the newcomers (well, some of them not so new :P): **Carlo** (and **Laura**), **Ángel**, **Rita**, **Wokke**, **Chris, Ragne**, **Shuyana**. Thanks for the dinners, the game nights, canoeing, the laughs, the pub quizzes, the birthdays, the excursions: In short, thank you for contributing to my life outside the PhD bubble and for bringing joy and novelty to my existence. Special thanks to **Chris** for rescuing me and saving my (failed) trips (so many times). To **Ruizhe**, for organizing the core sessions that kept our bodies fit and our minds safe during the pandemic, and to **Michele** for the after talks on our online gym.

Many thanks to all my hard worker students, **Stina**, **Ben**, **Adrián**, **Gonzalo**, **Amaya**, **Dion** and **Jenneke** for their interest and contribution to this thesis. I also thank you for letting me explore and improve my supervision skills and for the good moments outside the lab.

I really grateful to have been blessed with amazing flatmates that turned into friends. My PhD startup wouldn't have been the same without **Susanne**, **Moni**, and of course, my crazy, crazy **Maxime**. Our time together was short but intense, filled with long talks, brunches and barbeques. I also have a special mention to **Anja**, the perfect Swiss balance to the two other intense inhabitants of La Casa. Thank you for being such a good listener and friend.

Start a new adventure very far from home and from the amazing support I had there was not easy. Infinitas gracias a mis padres: **Javier y Olga**, que me han brindado la mejor educación y las mejores oportunidades y me han enseñado los valores en los que me reconozco: esfuerzo, sacrificio y honestidad. Desde que abandoné el nido con 17 años me habéis seguido ayudando y apoyando incondicionalmente en la distancia. Distancia que, desafortunadamente, se ha ido haciendo más y más grande. Vosotros os habéis tomado con filosofía eso de tener una hija bióloga y trotamundos, y lo habéis transformado en la excusa perfecta para que conocer Europa. También gracias por el apoyo a mi hermana **Cristina** y al resto de **mi familia** (de los que están y de los que faltan). Me he perdido mucho de vosotros, de vuestras vidas, durante estos 5 años, pero me seguís acogiendo como si hubiese estado allí todos los días. Mención especial para mis abuelitos, **Isabel** y **Alfredo**, que no entienden del todo lo que hago con esas bacterias y aguas residuales, pero me apoyan incondicionalmente.

To my friends back home: **Juan and Raúl** (now completely integrated into the wetsus among us community), to the girls **Patri, Leti and Rocío** and **María Jesús** for keeping me in the loop of your lives even though I am so far away. To **María**, for our long calls full of laughs and drama. You share with me the homesick feeling and understand the ups and downs of being an immigrant in a different land. To **Lorena and Sergio** for always making time to see me when I am back in Spain, even though we live far apart. To **David**, that rode the extra mile and visited me in Leeuwarden and then hosted me in Valencia.

To my Bio group of Dr Usal y tiral: **Noe, Diego, Victor, Sande, Usa, Patri, Peci, Sandra and Mario.** More than 10 years have passed since we said goodbye to Salamanca, yet you make me feel as if I was still there. Our talks are always light-hearted, and our reunions have been epic. I can´t wait for the next one. Thank you for the group cheers to each of our academic and personal achievements. I want to acknowledge the contribution of the many members of my other ¨support groups¨ **Mastermix** (special mention to **Sari**, **Diego O.** and **Lidia**) and to ¨**Salamanca y Pantallazos**", (always in my heart as ¨**El diario de Calde**¨). You also keep our friendship alive despite time and distance and provide me with many perfect cheering conversations to evade my busy mind, even in the worst moments. I would always regret missing the gatherings for being so far away

Pour ma belle famille Prot: **Dominique**, **Gérard**, **Simon**, **Aubin** et **Gautier**: vous m'avez accueilli comme votre fille et votre soeur. Avec vous j´ai decouvert une nouvelle façon de voir la vie, sans courir, profiter de chaque petit moment et pleine de joie. Je suis heureuse de vous avoir rencontré.

Karine, many people know the iron Russian girl that set the alarm off in the lab from time to time. Fewer lucky ones get to know the real you: generous, welcoming, inclusive, cheering, exceptional (pan)cakes maker. I am lucky you asked me to live with you, shared good and bad moments, long couch talks, many movies (well, you just saw the beginnings), dances, parties, trips. You have been an example of determination and of hard effort. Thank you for being my friend and for agreeing to be my paranymph

Thomas, ma poule, thank you for insisting. Your amazingly optimistic personality is invaluable to deal with my pessimistic mind. Next to you, everything seems more colourful, easier, lighter, funnier. Thank you for pushing me beyond my comfort zone and for showing me what life looks like without fear and with joy. You have been by my side throughout this long and difficult path, and you still chose me to continue a life together. Your support is all for me. I couldn't be luckier. I love you.

Gonçalo: You have been the person I have learnt the most from in this PhD. Not only technically but also personally. I hope you also took a bit from me with you after all this time. We are definitely very different in many aspects but at the same time understand each other incredibly well. Deep down, we share the same values, ethics, and passion for what we do, which is the secret to our friendship and working partnership. Thank you for having accepted doing a PhD at Wetsus. This thesis has a big piece of your soul and work.

About the author

Rebeca Pallarés Vega was born on September 27th in Palencia, Spain.

Her fascination for life sciences begun while watching nature documentaries. She soon knew that she wanted to learn everything about the diverse living organisms on this planet, so she enrolled in a BSc in Biology at the University of Salamanca (Spain).

Still broadly interested in many different biology disciplines, she decided to pursue an MSc. in Advances and Research in Microbiology at the University of Granada (Spain). During her master thesis, she studied the microbial diversity of goat dairy products and the biotechnological properties of starter strains for the dairy industry.

Rebeca moved to Leeuwarden in 2015 to do her MSc internship at Wetsus, where she first was acquainted with the antibiotic resistance problem. During her nine-month project, she investigated the resistant genetic profile of bacteria isolated from hospital wastewater.

Shortly after completing her internship, Rebeca was granted a PhD position at TU Delft and Wetsus. During her PhD, Rebeca has focused on evaluating the presence and removal of Antimicrobial Resistance Genes (ARGs) in wastewater and biosolids, trying to bring more insights into the role of operational parameters and ARG removal. In addition, Rebeca has investigated different conditions that could influence the spread of ARGs through horizontal gene transfer events in both *in vitro* and *in situ* set-ups. The results of her research are presented in this thesis.

In 2021, Rebeca participated in EU project REPARES on behalf of Wetsus, working to foster the cooperation of the academic institutions and stakeholders to tackle the AMR in wastewater. Currently, she continues her career at the University of Newcastle as PDRA in the project AMRflows UK-India, following up her research on plasmid-mediated dissemination of AMR and advising in the surveillance of ARGs in Indian water systems.

List of Publications

Pallares-Vega, R., Blaak, H., van der Plaats, R., de Roda Husman, A.M., Hernandez Leal, L., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2019. Determinants of presence and removal of antibiotic resistance genes during WWTP treatment: A cross-sectional study. Water Res. 161, 319–328. https://doi.org/10.1016/j.watres.2019.05.100

Pallares-Vega, R., Hernandez Leal, L., Fletcher, B.N., Vias-Torres, E., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2021. Annual dynamics of antimicrobials and resistance determinants in flocculent and aerobic granular sludge treatment systems. Water Res. 190, 116752. https://doi.org/10.1016/j.watres.2020.116752

Pallares-Vega, R., Macedo, G., Brouwer, M.S.M., Hernandez Leal, L., van der Maas, P., van Loosdrecht, M.C.M., Weissbrodt, D.G., Heederik, D., Mevius, D., Schmitt, H., 2021. Temperature and Nutrient Limitations Decrease Transfer of Conjugative IncP-1 Plasmid pKJK5 to Wild *Escherichia coli* Strains. Front. Microbiol. 12. https://doi.org/10.3389/fmicb.2021.656250